Prokaryotic Production of Human Immunodeficiency Virus Type 1 Subtype C Tat, Nef and Reverse Transcriptase and Investigation of Antibody Responses to these proteins in HIV-1 Infected Individuals as well as Macaques Vaccinated with SAAVI DNA-C/C2 and SAAVI MVA-C.

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In fulfilment of the requirements for the degree:
M.Sc (Medical Virology)

Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

Date of Submission: January 2010
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ACKNOWLEDGEMENTS

Firstly, I would like to thank God Almighty for standing by my side during the difficult times of the past two years and making it possible for me to see this thesis through to completion. I would not have made it this far without Him.

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this thesis possible:

My supervisor, **Prof Anna-Lise Williamson,** for giving me the opportunity to work in her lab and for all her time, input and invaluable support. My co-supervisors **Dr Nyasha Chin'ombe** and **Dr Gerald Chege** for having an 'open door' policy with invaluable assistance on the bench and for all their time, input, support and advice. Without all of you, this thesis may have not seen the light of the day.

Ros Chapman and **Helen Stutz**: 22 months ago if you asked me "Who is my mom?" I would have said "**Martha**". Today I would say "**Martha**" and her two guardian angels, Ros and Helen who gave me a reason to smile and made each and every day in the lab the best I could ever wish for. They gave me hope and a shoulder to cry on when the world threw its worst at me. **Ros** and **Helen**, I cannot imagine where I would have been without you. But that's what mothers do. **Ros**, much appreciation goes to you for your help in the quest to use the Western blot apparatus so that I was able to get "TANERT" in a good banding pattern.

Nicolene - Nicky. You are the best "sister" I could ever wish for. Your ever-smiling face always kept my spirits up.

Prisca, **Olivia**, **Mariba, Daniel, Jack, Andile, Zizipho, Koushik and Hayley:** for making life inside and outside the lab fun and enjoyable. You are the best office mates I could ever wish for. **Daniel**: for your help and time in answering every question I have ever asked you. **Olivia** and **Zenda**: for your support and proof-reading my thesis. **Olivia**, without you the typographical errors would have been significantly higher. **Nobuntu** and **Bruce**: for your invaluable help during clinical experiments. **Lycias**: for your help with statistical analysis.

To my mom, **Martha**, my siblings **(Punky, Tebogo, Sinah, Kgopotso, Maite, Ouma and Kgothatso)** and cousin **(Lucas)** to whom I dedicate this thesis. I thank you all for your love, support and patience.

Joseph, words are not enough.

Special thanks to the **NRF**, **PRF** and **UCT** for funding my study.

DECLARATION

I, Mankgopo M. Kgatle, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other university.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
α	alpha
a.a	Amino acid(s)
A_{260}	Absorbance at 260
A_{280}	Absorbance at 280
AIDS	Acquired immunodeficiency syndrome
Amp R	Ampicillin resistance
APS	Ammonium persulphate
Arg	Arginine
Asp	Aspartic acid
ß	Beta
µ	Micro
µl	Micro litres
µg	Microgram
Axnm	Absorbance at x-nm
bp	Base pairs
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CA	Canada
CD4	Cluster of differentiation 4
CCR5	CC Chemokine receptor 5
CXCR4	CXC Chemikine receptor 4
CA	Capsid
cm	Centimetre
CTL	Cytotoxic T lymphocytes
COOH	Carboxyl terminal
dam	DNA adenine methylase
DC	Dendritic cells
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FIV	Feline immunodeficiency virus
g	Gram
GFP	Green fluorescence protein
IPTG	Isopropyl-β-D-thio galactosidase
IgG	Immunoglobulin g
INF-γ	Interferon-gamma
Kb	Kilobase
kDa	Kilo-dalton
L	Litre
LCMV	Lymphocytic choriomeningitis virus
M	Molar
MA	Matrix
mg	Milligram
Ml	Millilitre
mM	Millimolar
NaOH	Sodium hydroxide
NBT-	Nitro blue tetrazolium chloride
NC	Nucleocapsid
Nef	Negative factor
ng	Nanograms
NH_2	Amino terminus
Ni-NTA	Nickel Nitrilotriacetic acid
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
pmol	Picomoles
phoP/Q	Phosphate metabloism
REV	Regulator of virion
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Reverse transcriptase
RNase	Ribonuclease
RRE	Rev response element
SA	South Africa
SAAVI	South African AIDS Vaccine Initiative
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
Tat	Transcription activator
TAR	Trans-acting responsive region

HIV	Human Immunodeficiency Virus	U	Unit
His	Histidine	Vif	Viral infectivity factor
HPLC	High performance liquid chromatography	Vpu	Viral protein U
HPV	Human papilomavirus	Vpx	Viral protein X
HRP	Horseradish peroxidase	UK	United Kingdom
Gly	Glycine	USA	United States of America
GST	Glutatjione-S-Transferase	UV	Ultra-violet
gp120	Envelope glycoprotein 120	V	Voltage
gp41	Envelope glycoprotein 41	VL	Viral load
gp 160	Envelope glycoprotein 61	v/v	Volume per Volume
H_2O	Water	w/v	Weight per Volume

PUBLICATION AND CONFERENCE POSTER PRESENTATION

1. **Kgatle M**, Williamson A-L and Chin'ombe N, Expression of HIV-1 subtype C Tat and Nef in *Salmonella* vaccine vector. *2008. AIDS Research and Human Retroviruses* 24 (S1), pp88 (Published abstract)
2. **Kgatle M**, Williamson A-L and Chin'ombe N, Expression of HIV-1 subtype C Tat and Nef in *Salmonella* vaccine vector, AIDS Vaccine 2008 Conference in Cape Town, 13-16 October, South Africa, (Poster presentation).

ABSTRACT

The prevalence of Human immunodeficiency virus (HIV)/Acquired immune deficiency syndrome (AIDS) continues to increase worldwide, with HIV-1 subtype C being the most predominant subtype in South Africa. This study focused on cloning, expression and purification of HIV-1 subtype C Tat, Nef and RT proteins from recombinant *Salmonella enterica* serovar Typhimurium. The resultant purified Tat, Nef and RT proteins were used as antigens to investigate the prevalence of antibodies using Western blot analysis against HIV-1 subtype C Tat, Nef and RT proteins in sera of South African HIV-1 infected individuals sampled at different stages of disease. Serum samples from rhesus macaques vaccinated with candidate HIV-1 vaccines (SAAVI DNA-C/C2 and SAAVI MVA-C) expressing HIV-1 subtype C proteins, Gag, RT, Tat, Nef and Env were also tested for HIV-1 antibody responses.

HIV-1 *tat, nef* and *rt* genes were cloned into prokaryotic pGEM expression vector to construct pGEM+Tat, pGEM+Nef and pGEM+RT, respectively. The pGEM+Tat, pGEM+Nef and pGEM+RT were transformed into attenuated strain of *Salmonella enterica* serovar Typhimurium and recombinant Tat, Nef and RT proteins were extracted from the transformants. High levels expression of recombinant Tat, Nef and RT proteins were confirmed on SDS-PAGE gels Western blots. Purification of these proteins was a problem so the project was continued with His-tagged proteins.

The recombinant Tat, Nef and RT proteins were purified from *Salmonella enterica* serovar Typhimurium transformed with plasmids contained HIV-1 *tat, nef* and *rt* genes in-frame with His-tag coding sequence. SDS-PAGE and Western blot showed that recombinant proteins were purified at high levels especially for Tat and Nef protein. The recombinant Tat and RT proteins could only be purified only under denaturing conditions because they are insoluble proteins and they can only purified as inclusion bodies by solubilizing with denaturant. Recombinant Nef protein is expressed as soluble protein and thus it was able to be purified under both denaturing and native conditions. However, the bulk of the Nef protein was expressed as inclusion bodies.

The purified Tat, Nef and RT recombinant proteins were used as antigens to investigate the prevalence of HIV-1 antibodies in sera of HIV-1 infected people. There was no reactivity in any of the 20 negative control sera in the Western blot assay. Analysis of 481 sera from HIV-1 infected individuals showed that the majority of serum samples had relatively high prevalence of anti-RT antibodies, ranging from 89.9% to 95% irrespective of the clinical stages. Anti-Nef antibodies were observed in 47.4% of individuals tested and there was a strong association between the prevalence of these antibodies with the clinical stage of CD4 count range of 201 to 499 cells/µl. The prevalence of anti-Tat antibodies was less frequent and only 7.5% of serum samples had anti-Tat antibodies. There was no association of anti-Tat and anti-RT antibodies with viral load or CD4 counts in sera of HIV-1 infected individuals. Altogether, 92% of serum samples tested had positive antibody responses to one or more antigens, indicating a good performance for the Western blot detection method.

The prevalence of antibody response against purified HIV-1 subtype C antigens (Tat, Nef and RT) was also investigated in eleven sera of macaques vaccinated with SAAVI DNA-C or SAAVI-DNA-C2 and SAAVI MVA-C. Western blot assays showed that 36%, 27% and 40% of serum samples from vaccinated rhesus macaques had detectable antibodies against HIV-1 subtype C Tat, Nef and RT antigens, respectively. The prevalence of antibody responses to Tat, Nef and RT antigens in the sera of macaques is lower relative to the prevalence of antibody responses in the sera of HIV-1 infected individuals. A possible explanation is that the immune system of HIV-1 infected people is continuously exposed to viral antigens, thus resulting to high level of detectable anti- Tat, -Nef and -RT antibodies. In contrast, macaques were exposed to HIV-1 antigens expressed by the DNA and MVA vectors for only five times through vaccinations. However, these results indicate that Western blot assay based on purified HIV-1 Tat, Nef and RT proteins may be a useful research tool for detection of antibody responses to corresponding vaccine immunogen in macaque sera.

In conclusion, HIV-1 subtype C Tat, Nef and RT were successfully purified from *Salmonella enterica* serovar Typhimurium. The purified Tat, Nef and RT recombinant antigens were antigenically recognised by anti-Tat, anti-Nef and anti-

RT antibodies in the sera of HIV-1 infected individuals and rhesus macaques vaccinated with candidate HIV-1 vaccines. Application of these purified proteins as reagents in a Western blot assay to detect antibodies in serum samples of HIV-1 subtype C positive individuals demonstrated an association between the prevalence of anti-Nef antibodies and CD4 count range of 201 to 499 cells/µl. In addition, the detection of antibody responses to purified Tat, Nef and RT antigens in the sera of vaccinated macaques suggests a possible application of these purified proteins in HIV vaccine research and improvement of existing Western blot-based HIV diagnostic kits.

CHAPTER 1

LITERATURE REVIEW

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1 GENERAL INTRODUCTION

Human immunodeficiency virus (HIV) was first identified in the early 1980s and has since been classified as one of the greatest health concerns globally (Barre-Sinoussi *et al.*, 1983). HIV is member of the genus Lentivirus belonging to the family *Retroviridae* (Barre-Sinoussi *et al.*, 1983). The virus is transmitted from one individual to another through blood or blood products (e.g blood transfusion) and unprotected sexual intercourse. HIV can also be transmitted through breast feeding in 10% to 20% of exposed infants and through mother-to-child transmission during birth in 15% to 30% of children born by HIV-infected mothers who do not receive anti-retroviral drugs (Coovadial *et al.*, 2007; Embree *et al.*, 2000; Jiang *et al.*, 2006). There are two types of HIV, namely HIV-1 and HIV-2. HIV-1 is the more pathogenic of the two types and is more common worldwide. HIV-1 infection in sub-Saharan Africa is responsible for greater than 50% of HIV-1 infections worldwide (Heeney *et al.*, 2006). HIV-1 and the simian immunodeficiency virus of chimpanzees (SIVcpz) share a common phylogenetic lineage, as HIV-1 originated from Western and Central African chimpanzees, *Pan troglodytes troglodytes*. HIV-2 is closely related to SIVmac and SIVsm that infect macaques and sooty mangabey monkeys, respectively (Rambaut *et al*., 2004).

1.1 THE STRUCTURE AND REPLICATION OF HIV-1

Two identical copies of a single stranded RNA of 9.5 kilobases (kb) is encapsidated in the HIV particle consisting of a capsid, matrix and envelope (Figure 1.1) (Muesing *et al.*, 1987; Giri *et al.*, 2004). At the virion surface there are envelope spikes with each spike representing a trimer of the two glycoproteins, gp120 and gp41 (Foster *et al.*, 2001). Gp120 and gp41 are generated through proteolytic processing of the precursor gp160 and makes up the outer surface and the transmembrane envelope, respectively (Figure 1.1).

During HIV infection, glycoprotein gp120 interacts with the host cell, CD4 receptor (Fig 1.2). This interaction causes the gp120 structure to undergo a conformational change and be able to bind either co-receptor CCR5 or CXCR4 depending on the virus. This subsequently results in fusion of the viral membrane with the membrane of the cell to be infected (Peterlin and Trono, 2003). Once the virus is inside the cell,

reverse transcriptase converts RNA to double-stranded DNA. The virus encoded enzyme integrase, allows integration of the viral cDNA with the cells' own DNA (Peterlin and Trono, 2003). Division of the HIV infected cell leads to expression of viral genes. During the budding stage, the viral Gag and Gag-Pol polyproteins assemble at the cell membrane with the viral RNA and form immature virions. The pr55gag polyproteinis cleaved by the viral protease enzyme into the matrix, nucleocapsid and capsid proteins (Peterlin and Trono, 2003) resulting in the formation of mature HIV with a condensed core as illustrated in Figure 1.1.

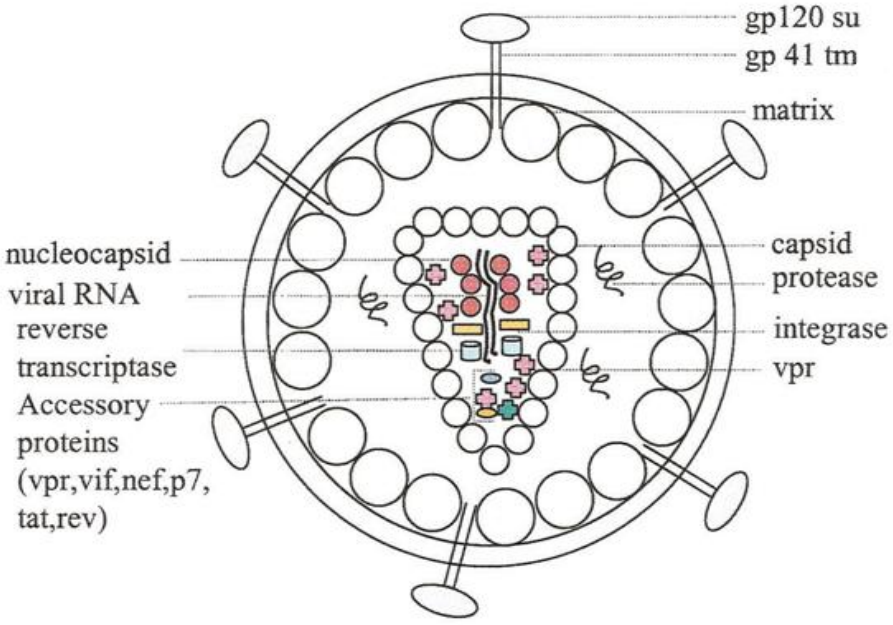

Figure 1.1: The structure of HIV (Giri *et al.*, 2004). The structure shows structural components of the virus including matrix, capsid, nucleocapsid, viral enzymes (reverse transcriptase, protease, and integrase), and accessory proteins (Vpr, Vpu, Nef, p7, Rev and Tat), su: surface, tm: transmembrane.

1.2 GENOME ORGANIZATION

The HIV genome is made up of nine open reading frames encoding three groups of viral proteins and all are identified in Figure 1.3. Structural proteins are encoded by *group antigen gene* abbreviated as *gag* and *env* (envelope). The *gag* gene encodes p55 precursor of the matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6 protein. The *env* gene encodes the gp160/gp140 precursor of surface glycoprotein gp120 and transmembrane gp41 glycoprotein (Frankel and Young, 1998). The *pol* gene encodes p64 and p53, which make up protease (p11), RT (reverse transcriptase; p66 and p51) and integrase (p34). Regulatory proteins include Tat (transcription

activator) and Rev (regulator of virion expression) and they regulate the viral gene expression (Holguin *et al*., 2001; O'Brien *et al*., 1997). Rev protein is a 13-19 kDa protein which allows transport of the late unspliced and partially spliced mRNA from the nucleus to the cytoplasm facilitating the expression of structural proteins (Pollard and Malim, 1998). Accessory proteins of HIV are also known as virulence factors and these proteins include Nef (Negative regulatory factor), Vif (Viral infectivity factor), Vpr (Viral protein), Vpu (Viral protein U, for HIV-1) or Vpx (Viral protein X, for HIV-2) (Piquet and Trono, 1999). These proteins enhance HIV-1 replication both in cell culture and *in vivo* (Kestler *et al*., 1991). This review will provide a detailed description of Tat, Nef and RT as they are the main focus of this study.

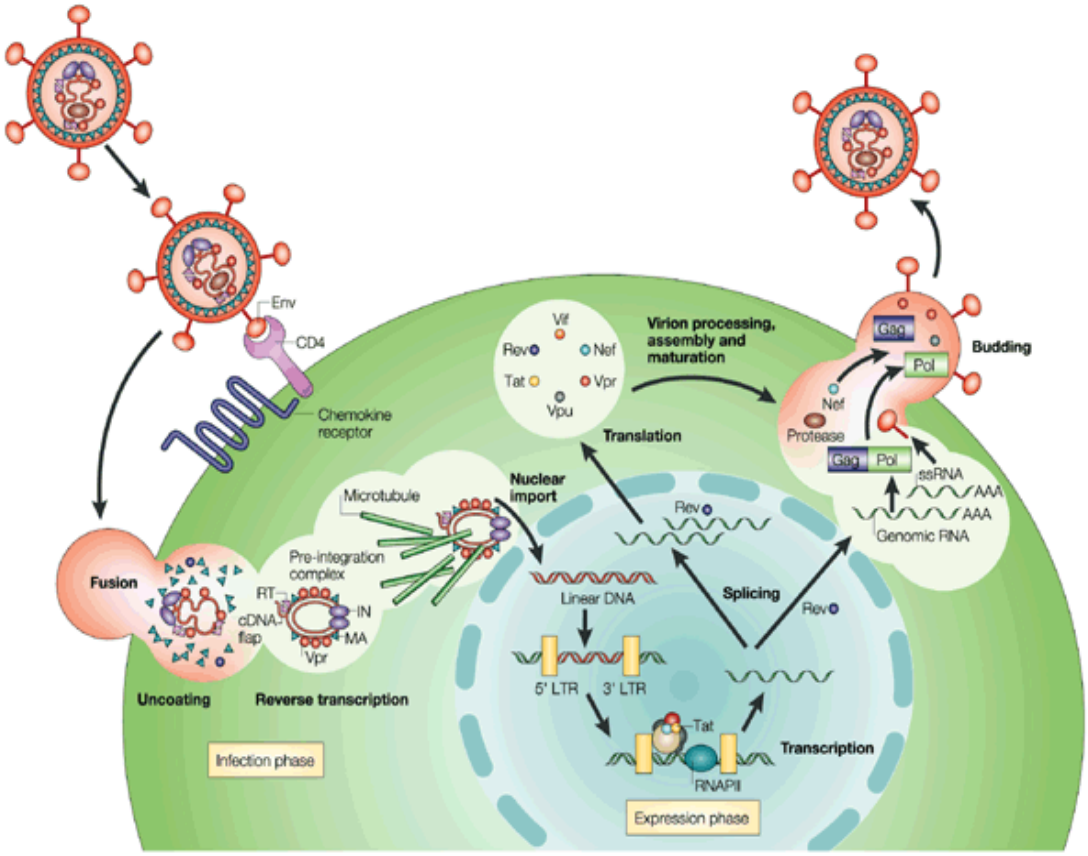

Figure 1.2: The replicative cycle of HIV (Adapted from Peterlin and Trono, 2003). HIV-1 gp120 binds the human cell, CD4. The gp120-CD4 complex makes the gp120 structure undergo conformational change allowing binding of CCR5 or CXCR4 which leads to viral fusion. The viral capsid is uncoated and enters the nucleus where reverse transcriptase convert ssRNA into dsDNA, The dsDNA is integrated and becomes provirus. Tat is expressed and increases the transcription level of the provirus. Rev transport spliced and unspliced genome transcripts from the nucleus to the cytoplasm where viral structural and enzymatic proteins are synthesised. Nef facilitates viral assembly.

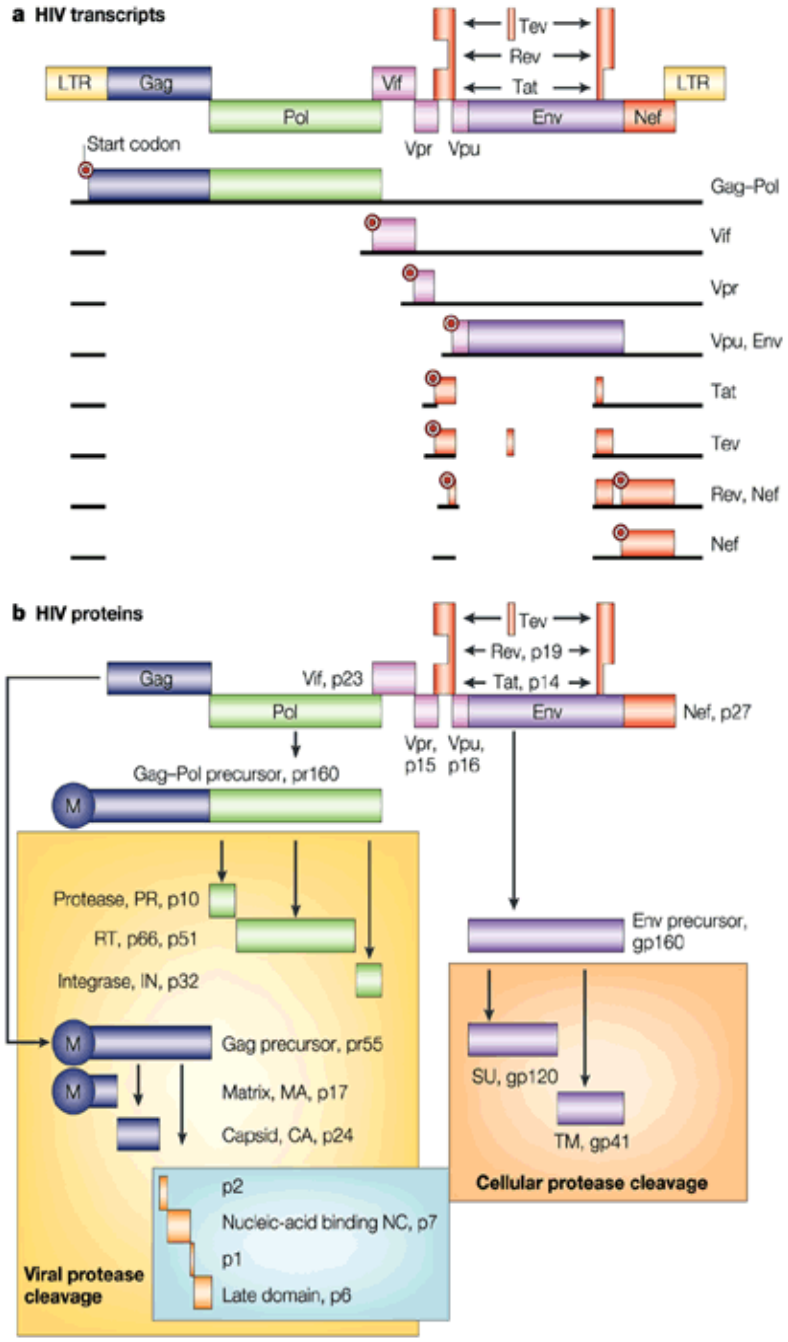

Figure 1.3: HIV-1 genomic organisation, transcripts and proteins (Peterlin and Trono, 2003). The 10-kb viral genome contains open reading frame for 16 proteins, synthesised from at least ten transcripts. Black lines represent unspliced and spliced transcripts, above which coding sequences are given, with the start codons indicated. Above the encoding sequences, all singly spliced transcripts are shown. Tat requires Rev for their export from the nucleus to the cytoplasm. The RNA targets for Rev and the Rev response element (RRE) are contained in the gene encoding the envelope protein (Env).

1.2.1 Tat Protein

1.2.1.1 Structure of Tat protein

The *transcription activator* (*tat*) protein binds to its response element located in the HIV-1 5' long terminal repeat (LTR) and is found in the nucleolus or nucleus (Bres *et al*., 2002; Jeang *et al*., 1999). It is usually expressed soon after viral infection in HIV infected patients (Wu and Marsh, 2001). Tat is a 14-16 kilodalton (kDa) protein encoded by the *tat* gene of the HIV-1 genome (Rana and Jeang, 1999) and it occurs as two isoforms: a smaller 14 kDa Tat protein encoded by the first exon and a full-length 16 kDa protein encoded by the next two exons (Blazquez *et al.,* 1999; Goldstein, 1996).

The first exon, p14 is located in the central region of the viral genome and it encodes the 72 amino acids of Tat protein. Tat can be divided into amino terminal and basic domain regions and a cysteine rich region (aa 22-37) with 7 cysteines in 16 residues (Orsini *et al.,* 1996). The amino (NH2) terminal domain (aa 1-21) is rich in proline and acidic residues and is essential for Tat function. A highly basic region is located at position 49-72 with 2 lysines and 6 arginine in 9 residues (Bres *et al*., 2002; Van Duyne *et al*., 2008; Vendeville *et al*., 2004). This region is important for nuclear localisation, facilitating the transport of Tat into the nucleus for binding to the transactivating response element (TAR) (Bres *et al.,* 2002; Vendeville *et al*., 2004). TAR is a 55-nucleotide RNA stem loop structure located at the 5' end of HIV-1 transcripts which acts to increase the level of viral transcription (Bres *et al*., 2002).

The second exon, p16 encodes aa 73-86, and overlaps with the open reading frame of the envelope gene (Blazquez *et al*., 1999; Cui *et al*., 2004; Jeang *et al*., 1999). This exon encodes the extracellular matrix, carboxyl terminus (COOH) which is encoded by amino acids 14 to 32 and carries the highly conserved Arg-Gly-Asp (RGD) sequence (Blazquez *et al.,* 1999; Chang *et al*., 1997). The RGD conserved region is involved in binding to the cell surface intergrins (Brake *et al*., 1990; Ruoslahti, 1996).

1.2.1.2 Functions of Tat protein

Tat protein interacts with TAR by recruiting cyclin T1 and cyclin dependent kinase 9, a complex that phosphorylates the carboxy-terminal domain of RNA polymerase II. This process stimulate the viral gene expression that promote HIV replication and transmission (Berkhout *et al*., 1989; Bres *et al*., 2005; Chang *et al.,* 1994; Deng *et al.,* 2000; Jones and Peterlin, 1994). The presence of Tat facilitates expression of structural genes (*env, gag,* and *pol*) in order for viral replication to proceed (Fisher *et al*., 1986). Although the virus is still able to infect CD4+ cells in the absence of Tat protein, it cannot undergo successive rounds of replication (Fisher *et al*., 1986; Richardson *et al*., 2003). This abortive replication occurs because Tat protein regulates efficient elongation of viral transcripts as well as viral infectivity by involving factors such as post transcription, transport of mRNA and translation (Feng and Holland, 1988; Peterlin *et al.,* 1986). This process enhances the progression of AIDS disease (Richardson *et al*., 2003).

1.2.1.3 Expression and purification of Tat protein

Recombinant Tat protein has been produced in different expression systems including *E. coli* strains (Fanales-Belasio *et al*., 2002b; Larder *et al.,* 1987; Ma *et al.,* 2006). The production of purified Tat protein has been achieved by using different purification techniques such as heparin-affinity chromatography and high performance liquid chromatography (HPLC) and affinity chromatography (Blázquez *et al*., 1999; Fanales-Belasio *et al.,* 2002b; Ma *et al.,* 2006; Park *et al*., 2002; Scheich *et al*., 2003). Due to the solubility of protein, Tat can be purified as inclusion bodies under denaturing conditions using urea. The protein yield of Tat protein may result in 98% purity (Ma *et al.,* 2006; Park *et al*., 2002). Tagging of Tat and other protein with His- and GST-tags is most commonly used and this process facilitates purification of proteins by affinity chromatography using Ni-NTA and glutathione agarose, respectively (Scheich *et al*., 2003). However, a study by Scheich *et al.,* 2003 showed that His-tag fusion protein with Ni-NTA agarose produces higher yield of the purified protein compared to GST-tag using glutathione agarose. Using of GST-tagged protein requires the use of glutathione agarose with reduced glutathione as it can affect target proteins containing disulphide bonds (Scheich *et al*., 2003).

1.2.1.4 Antibody responses to recombinant Tat antigen

Tat protein is expressed soon after HIV infection before expression of structural genes and viral integration and plays a functional role in the viral life cycle (Ma *et al*., 1997; Jones and Peterlin, 1994). Recent studies conducted in Uganda and South Africa showed that immunogenic regions of Tat are well conserved among different HIV-1 subtypes particularly subtypes B and C (Butto *et al*., 2003; Fanales-Belasio *et al*., 2002a). A study by Butto *et al.* (2003) observed a prevalence of 50% of antibody responses to Tat protein in individuals with HIV-1 infection from Uganda. The persistence of anti-Tat antibodies in HIV-1 infected individuals is associated with slow progression of HIV-1 disease (Butto *et al.,* 2003; Re *et al.,* 2001; Richardson *et al*., 2003).

In another study, Demirhan *et al,* (1999) used enzyme-linked immunosorbent assay (ELISA) to investigate the detection of antibodies to HIV-1 Tat in HIV-infected individuals with or without Kaposi's sarcoma (KS). Their results showed that 16% of individuals with HIV-1 and KS had anti-Tat IgG antibodies in their sera. However, no anti-Tat antibodies were observed in HIV-negative individuals and KS patients who were HIV-1 negative. This data suggests that the presence of Tat antibodies could be involved in pathophysiology of HIV-1 infection and may be associated with rapid disease progression (Demirhan *et al*., 1999), which is contrary to the findings of other studies (Belliard *et al.,* 2003; Butto *et al.,* 2003; Opi *et al.,* 2002; Re *et al.,* 2001; Rezza *et al*., 2005; Richardson *et al.,* 2003; Zagury *et al.,* 1998).

In a study to evaluate the prognostic value of anti-Tat antibodies, Rezza *et al*. (2005) used HIV-1 subtype B Tat protein to detect the presence of anti-Tat antibodies in the sera of HIV-1 infected patients. They found that 11.5% of the sera had anti-Tat antibodies. There was strong association of anti-Tat antibodies with slow disease progression (Rezza *et al.,* 2005). HIV-1 infected patients with high level of anti-Tat antibodies showed long-term non-progression to AIDS compared to patients with low level of anti-Tat antibodies (Belliard *et al.,* 2003; Butto *et al.,* 2003; Opi *et al.,* 2002; Rezza *et al.,* 2005; Richardson *et al.,* 2003; Zagury *et al.,* 1998). This finding was however, in opposition to the findings reported by Wieland *et al*. (1990) and Krone *et al*. (1988) in which they found that 16% to 40% of the sera from HIV

infected individuals had anti-Tat antibodies and this increased in patients with higher viral load and lower CD4 counts.

The examples cited above illustrate that antibodies to Tat protein are present in the sera of HIV-1-infected individuals although they are variably detected in different populations. In addition, these studies drew differing conclusions regarding the association between the detection of anti-Tat antibodies and the rate of disease progression. It is possible that the different findings were the result of variable sensitivities and specificities of the methods of detection, suggesting that the detection of anti-Tat antibodies is dependent on the HIV-1 subtype from which the Tat protein was derived. Incorporating Tat protein derived from the HIV-1 subtype which is most prevalent in the target population in anti-Tat antibody detection may be expected to produce high performance of the detection method. Despite these findings, there is no similar information reported in regard to the detection of anti-Tat antibodies in HIV-1 subtype C infected patients. Moreover, there is no clear evidence of association of the presence of anti-Tat antibodies with the disease progression in this population with subtype C infection.

1.2.2 Nef Protein

1.2.2.1 Structure of Nef protein

The *nef* gene is located on the 3' end LTR of the viral RNA genome (Chaudhuri *et al.,* 2007; Niederman *et al.,* 1993). It encodes the 27 kDa Nef protein (also called Negative factor), which is a negative regulatory factor of HIV-1 replication (Chaudhuri *et al.,* 2007; Terwiliger *et al.,* 1991; Walker *et al.,* 2007). Nef is expressed at an early stage of HIV-1 infection (Arold and Baur, 2001; Klotman *et al.,* 1991). Unlike Nef of HIV-2 and SIV (250 amino acids long), HIV-1 Nef is slightly smaller in length of 206 amino acids (Niederman *et al.,* 1993; Wei *et al.,* 2003). Nef protein occurs as two isoforms; full-length myristylated amino terminal p27 protein and truncated p25 protein generated from the second start codon, so that it lacks the first 18 amino acids (Arold and Baur, 2001; Fackler *et al.,* 1997). The p27 isoform is myristylated and as a result, it localises to the cellular membranes and is phosphorylated on serine and threonine residues. Due to the lack of myristylation of p25, it is only found in the cytoplasm (Yu *et al.,* 1992).

1.2.2.2 Functions of Nef protein

Nef protein plays an important role in the viral life cycle and pathogenesis of HIV and SIV infections *in vivo* through a number of ways (Moureau *et al*., 1999; Wei *et al*., 2003). Nef protein has been shown to down-regulate the HIV-1 receptors, CD4 and CD28. This process prevents superinfection and promotes viral replication (Michel *et al*., 2005; Miller *et al.,* 1994; Piquet *et al.,* 1999; Wei *et al*., 2003). Nef protein also down-regulates the expression of MHC Class1 during early HIV infection and facilitates escape of infected cells from the Cytotoxic T-lymphocytes (CTL) response (Schnidler *et al*., 2006; Wei *et al.,* 2003). Finally, Nef protein interacts with tyrosine and serine/threonine kinases to alter the activation state of cells leading to efficient proviral DNA synthesis and enhanced virion infectivity (Greenway *et al.,* 2003; Linnemann *et al*., 2002; Piquet *et al.,* 2003). HIV-1 Nef protein derived from subtypes B and C is highly immunogenic and well conserved. Both subtypes B and C have both B and T-cell epitopes which act to maintain viral function and fitness (Azad, 2000; Frahm *et al*., 2004; Masemola *et al*., 2004; Mashishi *et al.,* 2001).

1.2.2.3 Expression and purification of Nef protein

Recombinant Nef protein has previously been produced in a variety of host cell systems such as *E. coli* cells, yeast, insect cells lines, plants (Tobacco Mosaic Virus), human and mammalian cells (Marusic *et al*., 2007; Moureau *et al.,* 1999; Vermasvuori *et al.,* 2009). Affinity chromatography using His- and GST-tag fusion protein, pseudoaffinity Cibacronble F3GA column and AcA54 gel-filtration column have been successfully used to obtain purified Nef proteins (Federico *et al.,* 2001; Finzi *et al*., 2003; Wolber *et al*., 1992). Nef protein is very soluble and it can be purified at a high purity (95%) under both native and denaturing conditions using affinity chromatography (Federico *et al.,* 2001; Finzi *et al*., 2003). However it has also been reported that production of this protein in *E. coli* can result in the formation of inclusion bodies which are cytoplasmic aggregates that represent the presence of misfolded protein (Vermasvuori *et al.,* 2009). However, despite the disadvantages of *E.coli* production which include lack of secretion of the Nef protein, Nef being found in inclusion body and the need for purification to remove bacterial endotoxins and

other proteins *E.coli* production was still more efficient and cost effective than production in yeast or insct cells (Vermasvuori *et al.,* 2009).

1.2.2.4 Antibody responses to recombinant Nef antigen

As mentioned earlier, Nef is expressed at an early stage during the HIV life cycle and plays an important role in the pathogenesis of HIV (Fuji *et al.,* 1996; Otake *et al.,* 1994). Bahraoui *et al,* (1990) investigated whether detection of anti-Nef antibodies can serve as an early marker of HIV infection. HIV-1 Nef derived from subtype B (*LAV $_{Bru}$ Strain*) was expressed and purified from *E. coli* by ultra filtration. Purified Nef protein was used to investigate the prevalence of anti-Nef antibodies in the sera of HIV-1 infected patients using radioimmunoassay (RIA), Western blot and ELISA (Bahraoui *et al.,* 1990). It was found that 30% to 70% of the sera sampled from HIV-1 infected patients with various CD4 counts had anti-Nef antibodies. These results indicated that the presence and/or detection of anti-Nef antibodies were not consistent enough to be used as an early marker of HIV infection (Bahraoui *et al*., 1990).

Chen *et al.* (1999) investigated the correlation of anti-Nef antibody with AIDS disease progression. They conducted a follow up study on sera obtained from 174 HIV-1/ AIDS patients before and after they received 1 year of anti-retroviral treatment. This study employed a Western blot assay with purified recombinant Nef protein and showed that 77% of the sera had anti-Nef antibodies. Chen *et al.* (1999) further showed that 21.1% of patients who initially showed no anti-Nef antibody response seroconverted (conversion of negative response to positive response) while 7.2% of patients who were positive to anti-Nef antibodies seroreverted (the reversion of positive response to negative response). This was an expected outcome due to anti-retroviral treatment. A high percentage of anti-Nef antibodies in the sera from HIV-1 infected patients regardless of their clinical stage has also been reported and it was strongly associated with slow disease progression (Wieland *et al*., 1990). The above studies used sera from HIV-1 subtype B infected individual. Similar studies using HIV-1 subtype C infected individuals have not been reported.

1.2.3 Reverse Transcriptase (RT) protein

1.2.3.1 Structure of RT protein

HIV-1 RT protein is encoded by the *pol* gene (Hang *et al.*, 2004). RT is a heterodimer protein composed of two subunits; a 66 kDa subunit (p66) having 560 amino acids and a 51 kDa subunit (p51) with 440 amino acids (Sluis-Cremer *et al.*, 2004). The p51 subunit is generated from p66 through proteolytic cleavage and lacks the ribonuclease H (RNase H) domain (di Marzo Veronese *et al.*, 1986). The p66 subunit has RNase H activity located within a domain of 120 amino acids that catalyses the degradation of the RNA strand of RNA/DNA hybrids (Hang *et al*., 2004; Telesnitsky and Goff, 1997). Both p66 and p51 subunits have the polymerase domains although the polymerase active site in the p51 subunit is not functionally active (Wang *et al.*, 1994). The p66 protein dimerises to produce p66/p66 homodimers that display DNA polymerase and RNase H activities (Restle *et al*., 1992). RT is composed of the DNA-binding cleft formed by four domains in both p51 and p66 subdomains (Figure 1.4). These include fingers, thumb, palm and connection (Jonckheere *et al.*, 2000; Sarafianso *et al.*, 2002). However, the p66 subdomain also has carboxyl terminal RNase H that serves as the fifth domain (Huang *et al*, 1998). The connection and palm domains are individually composed of three beta sheets with alpha helices and are important for the DNA polymerase catalytic activity (Frankel and Young, 1998). The thumb subdomain is composed of three alpha helices (Jonckheere *et al.*, 2000; Sarafianso *et al.*, 2002). The finger and thumb subdomains describe a deep cleft and are important for the polymerase active site of the p66 subunit which is defined by three catalytic aspartic acid residues (Asp110, Asp185, Asp186) that may play a role in binding metal ions (Frankel and Young, 1998; Olivares *et al.*, 1999).

1.2.3.2 Function of RT protein

RT transcribes single-stranded viral RNA template into double-stranded DNA through reverse transcription (Havlir *et al.*, 1996; Mitsuya, *et al*., 1990). This process is carried out by the active site of subunit p66 and is essential for HIV propagation (Havlir *et al.*, 1996; Mitsuya, *et al.*, 1990). The process of reverse transcription is error-prone and it leads to high viral mutation rate that can confer drug resistance (Havlir *et al.*, 1996; Mitsuya, *et al*., 1990). RT protein displays RNase H activity that

catalyses the degradation of the RNA strand of RNA/DNA hybrids required for reverse transcription (Hang *et al.*, 2004). The p51 domain acts as the binding site for the anti-codon and loop of tRNAs that serve as primers for the synthesis of the DNA template (Frankel and Young, 1998).

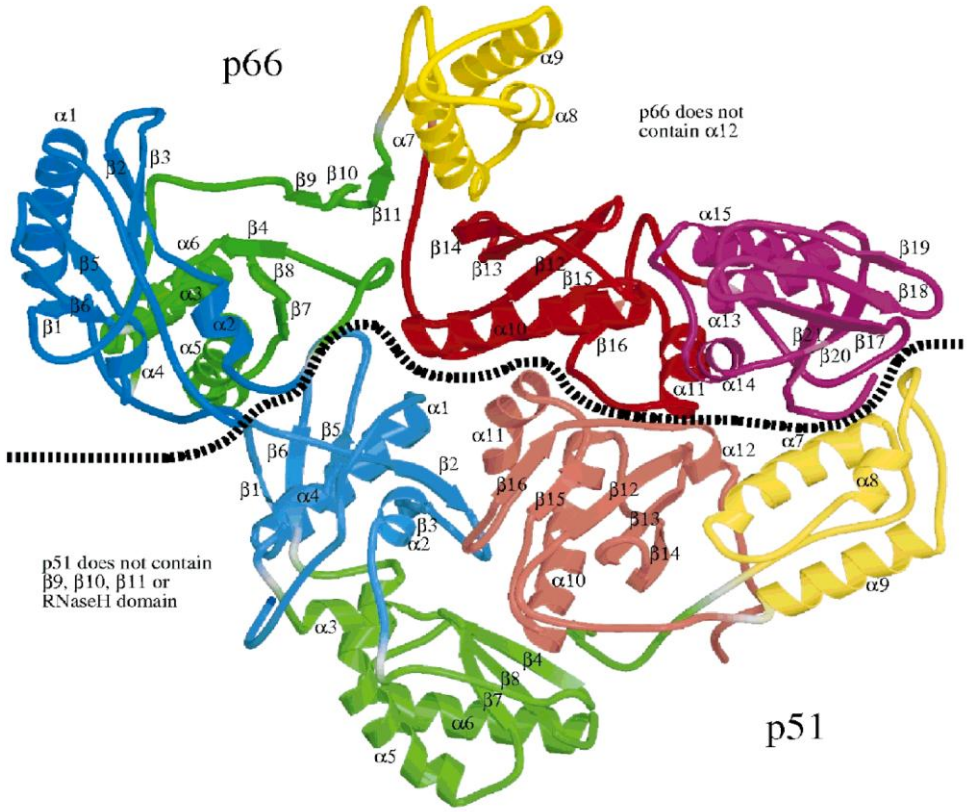

Figure 1.4: The three dimensional structure of HIV-1 RT protein (Adapted from Jonckheere *et al.*, 2000). The blue colour represents finger domains, yellow represents thumb domains, green represents palm domains, red represents the connections and purple represents RNaseH domains.

1.2.3.3 Expression and purification of RT protein

RT protein has been produced in *E. coli* using prokaryotic expression vectors (Fletcher *et al.*, 1996). The RT protein has been purified with SP-Sepharose, Affi-Gel Blue columns and with and/or without His-tag label by affinity chromatography (Fletcher *et al.*, 1996; Grüninger-Leitch, 1990; Hou *et al.*, 2004). RT protein is expressed as insoluble bodies and it can only be purified under denaturing conditions by solubilisation with urea (Rudolph and Lilie, 1996; Vallejo and Rinas, 2004). RT protein is rapidly degraded and thus using inhibitors such as

phenylmethanesulphonylfluoride (PMSF) during purification may aid in preventing the proteolytic degradation (Rudolph and Lilie, 1996).

1.2.3.4 Antibody responses to recombinant RT antigen

RT protein is routinely incorporated in screening and diagnostic tests. However, only few studies have reported on anti-RT antibodies in the sera of HIV infected individuals. RT is produced early in the life-cycle of HIV-1 infection and it catalyses reverse transcription of RNA into DNA to promote viral replication (Havlir *et al.*, 1996; Mitsuya, *et al*., 1990).

RT protein is highly immunogenic and it is consistently detected in the sera of HIV-1 infected patients irrespective of the clinical status (di Marzo-Veronese *et al.*, 1986). Using Western blot assay, 700 sera from HIV-1 subtype B infected individuals were tested for the prevalence of antibodies to RT (p66) in three different stages (asymptomatic, AIDS related complex (ARC) and AIDS) of the disease (DeVico *et al*., 1988). Nearly 80% of the sera had anti-RT antibodies regardless of the clinical stages of the disease (DeVico *et al.*, 1988).

In another study, Odawara *et al*. (1996) used a non-radioisotopic reverse transcriptase assay to investigate the prevalence of anti-RT antibodies in the sera of HIV-1 subtype B infected individuals. Their results showed that 83.6% of the sera tested had anti-RT antibodies. The prevalence of anti-RT antibodies was associated with slow disease progression in HIV infected patients (Odawara *et al.*, 1996). This was also shown by the high prevalence of anti-RT antibodies that protected HIV-1 infected individuals and reduced disease progression through inhibition of RT enzymatic activity (Jonckheer *et al*., 2000; Laurance *et al*., 1987).

1.3 HIV VACCINES

1.3.1 HIV vaccine pre-clinical development

The development of an HIV vaccine involves three general stages: discovery, pre-clinical development and clinical trials. Prior to clinical development, a vaccine must satisfy basic requirements to ensure safety and immunogenicity. Initial immunogenicity is tested in small animals such as mice and guinea pigs (McCune, 1991). Vaccines must also show immunogenicity and safety in non-human primate

species such as chimpanzees and macaques (cynomolgus, rhesus and pig-tailed) (Joag *et al.*, 2000; Kahn *et al*., 1998; Li *et al*., 2000). Non-human primates share many immunological characteristics (MHC antigens, T-cell receptors antigens, molecularly and biologically cytokines) with humans, making them the most suitable animal models for HIV studies (Joag *et al*., 2000; Li *et al*., 1992; van Maanen and Sutton, 2003). Among these species, only chimpanzees can be infected with HIV and were thus preferred as the most suitable model for HIV studies. There are however, disadvantages of working with chimpanzees because they rarely show AIDS symptoms, they are scarce and endangered species, and it is very expensive to work with them (Gardner *et al.,* 1990; Li *et al*., 1992; van Maanen and Sutton, 2003). Instead, macaques are being used since they can acquire a disease similar to AIDS when they are infected with SIV or when infected with recombinant SHIV (Mäkitalo *et al*., 2004). SHIV is a combination of HIV and SIV which mimics HIV infection (Mäkitalo *et al*., 2004). However, monkeys challenged with SHIV (SHIV89.6P) progress to disease faster than most humans which is why this strain is no longer used (Reimann *et al*., 1996). Another limitation of using monkey models is the fact that the vaccines must be modified so that they carry SIV genes (Li *et al*., 1992).

1.3.2 HIV vaccine strategies

Many different vaccine strategies are currently applied for the development of HIV vaccines. These strategies included using recombinant subunit, viral vector and DNA vaccines as vaccine delivery systems (Fernandez-Cruz *et al.,* 2003; Ohmit *et al*., 2008; Whatmore *et al*., 1995). For other human viruses such as polio, hepatitis A, rabies and influenza whole inactivated and live attenuated vaccines are the most common and successful vaccines (Fernandez-Cruz *et al.,* 2003). Live-attenuated vaccines include measles, rubella, polio and chicken pox (Daniel *et al.,* 1992; Whatmore *et al*., 1995). A possible complication associated with whole inactivated and live attenuated vaccines is their possible reversion to a virulent form as shown previously (Daniel *et al.,* 1992; Ohmit *et al.*, 2008; Whatmore *et al*., 1995). This is of particular concern with HIV because of the high mutation rate making these strategies too risky. Recombinant subunit vaccines are vaccines that use a specific purified antigen separated from virulent organism. These vaccines are being used against hepatitis B and human papillomavirus (Schellenbacher *et al.,* 2009; Schiller

et al., 2008). Although, subunit vaccines can be used to avoid risks associated with live attenuated and whole killed vaccines, they mainly elicit antibody responses and weak cell-mediated responses (Schellenbacher *et al.,* 2009; Schiller *et al.,* 2008). DNA vaccines have also been developed as HIV vaccines and they involve cloning of the desired gene into the bacterial plasmid (Castaldello *et al*., 2006; Darrah *et al.*, 2007; Kumer *et al*., 2006; Robinson *et al*., 2006). DNA based vaccines have been used in combination with subunit and viral vector vaccines for HIV studies. They have the ability to elicit robust CD8+ T cell responses (Castaldello *et al*., 2006; Darrah *et al.*, 2007; Kumer *et al*., 2006; Robinson *et al*., 2006).

1.3.2.1 Viral vectors

Viral vectors are used to deliver vaccine antigens to antigen-presenting cells (APC) (Catanzaro *et al*., 2006). Their most attractive feature is that they can induce antibody responses and cell mediated responses (CD8+ and CD4+) to recombinant antigens. Adenovirus 5 (replication-defective recombinant vector), ALVAC (derived from canarypox virus) and Modified Vaccinia virus Ankara (MVA) are good examples of such vectors and they have been used in several HIV studies (Catanzaro *et al*., 2006; Mwau *et al.*, 2004; Sandström *et al*., 2006; Trinvuthipong *et al*., 2004). These viral vectors can be used together and in combination with DNA vaccine vectors to boost immune responses and this process is known as heterologous prime-boost immunization (explained in detail in Chapter 5). The current study focuses on plasmid DNA vaccines boosted with MVA viral vector.

1.3.2.1.1 Modified Vaccinia virus Ankara (MVA)

MVA is an attenuated viral vector derived from Vaccinia virus (Hanke *et al*., 2005; Seth *et al.*, 1998). It has been used as a vaccine for small pox (Hanke *et al*., 2005). MVA has been used as an HIV vaccine vector in various animal models because of its ability to express high levels of foreign antigen and accept large inserts as well as its safety profile for use in humans (Cosma *et al.*, 2003; Meyer *et al*., 1991; Ramirez *et al*., 2000; Stittelaar *et al.*, 2002).

Hanke *et al*. (2005) demonstrated that MVA has the ability to induce robust of immune responses against vectored antigens. This was observed in mice vaccinated

with recombinant MVA expressing influenza virus haemagglutinin and nucleoprotein genes (Sutter *et al.*, 1994). In another study, Hirsch *et al.* (1996) compared the immunogenicity and protective efficacy of MVA-SIV and Wyeth-SIV (replication competent vaccinia strains) vaccines expressing SIVsm *gag-pol* in vaccinated macaques. Broad immune responses to Env and Gag-Pol proteins were detectable in MVA-SIV vaccinated macaques whereas vaccination with Wyeth-SIV resulted in low levels of vaccinia virus and SIV neutralising antibodies. The MVA-SIV vaccine prevented SIV infection in vaccinated macaques whereas the Wyeth-SIV vaccine did not (Hirsch *et al.*, 1996).

Recent study made in South Africa, showed that DNA and MVA vaccines expressing HIV-1 subtype C multigene polyprotein was highly immunogenic and generated high levels of HIV-specific CD4+ and CD8+ cells in mice, guinea pigs and baboons (Shephard *et al*., 2008; Burgers *et al*., 2008 & 2009). Similar results were obtained in rhesus macaques vaccinated with same vaccines (unpublished data). All the above examples indicated that MVA improves the immune response in animal models.

1.4 OVERALL OBJECTIVES OF THE PROJECT

HIV-1 Tat, Nef and RT proteins can be used to detect specific antibodies in the sera of HIV-1 infected patients. Previous studies showed that antibodies to HIV-1 Tat, Nef and RT were detectable in sera of HIV-1 subtype B infected patients at various quantities. Thus, this study aimed to clone, express and purify HIV-1 subtype C Tat, Nef and RT proteins. The first antibody study was performed using purified Tat, Nef and RT as antigens to investigate the prevalence of the antibody responses in the sera of HIV-1 subtype C infected patients. The second part of this thesis tested for antibodies in non-human primates vaccinated with two vaccines, funded by South African AIDS Vaccine Initiative (SAAVI). Therefore the sera from macaques vaccinated with the SAAVI DNA-C/C2 vaccine and boosted with SAAVI MVA-C were also tested for antibodies to Tat, Nef and RT.

The specific objectives of the project were:

- To clone and express HIV-1 subtype C Tat, Nef and RT proteins in *Salmonella enterica* serovar Typhimurium (Chapter 2).

- To purify His-tagged HIV-1 subtype C Tat, Nef and RT proteins from *Salmonella enterica* serovar Typhimurium for Western blotting (Chapter 3).

- To investigate the prevalence of HIV-1 Tat, Nef and RT antibodies in the sera of HIV-1 infected individuals as detected by Western blot (Chapter 4).

- To investigate whether there is an association between the prevalence of these antibodies and the clinical stage of AIDS disease (as categorized by CD4+ T cell count and plasma viral load) (Chapter 4)

- To investigate the prevalence of HIV-1 Tat, Nef and RT antibodies in the sera of macaques vaccinated with SAAVI-DNA and SAAVI-MVA expressing HIV-1 proteins (Gag, RT, Tat, Nef and Env) (Chapter 5).

CHAPTER 2

DEVELOPMENT OF RECOMBINANT *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM EXPRESSING HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C TAT, NEF AND REVERSE TRANSCRIPTASE

CONTENTS

2.1 INTRODUCTION

The genus *Salmonella* is made up of rod-shaped, gram-negative *enterica* bacteria that belong to the *Enterobacteriaceae* family (Miller *et al.,* 1995; Chang *et al.*, 1997b). The bacteria cause clinical syndromes such as typhoid fever and gastroenteritis in humans and animals after intake of contaminated water or food (Crump *et al.,* 2004; Voetsch *et al*., 2004; Foley *et al*., 2006; Morpeth *et al*., 2009). The *Salmonella* genus is divided into two species: *Salmonella enterica* and *Salmonella bongori* (McQuiston *et al.,* 2008, Lan *et al*., 2009). The *Salmonella enterica* (*S. enterica*) species is further classified into 6 subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *hautenae* and *indica* (Lan *et al.,* 2009). Each subspecies has several serovars. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (sometimes written simply as *Salmonella enterica* serovar Typhimurium) is a major nontyphoidal human pathogen worldwide (Gordon *et al*., 2008; Kingsley *et al*., 2009). In mice, this serovar causes typhoid-like symptoms and is therefore used as a mouse model for human typhoid fever caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*Salmonella enterica* serovar Typhi) (Santos *et al.*, 2001).

To develop vaccines against a number of *Salmonella enterica* species, mutations have been introduced into the genes that affect the metabolic pathways of the bacteria. The introduction of these specific mutations into genes such as *aroC/D (*aromatic amino acid synthesis), *htrA (*heat and oxidative stress*), pur (*purine biosynthesis), *phoP/Q* (phosphate metabolism) and *dam* (DNA adenine methylase expression) has been shown to attenuate the bacteria, thereby making them safe for use as vaccines (Castelli *et al.,* 2000; Chamnongpol *et al.*, 2003; Curtiss, 1993; Heithoff *et al.,* 2001; Mutunga *et al.,* 2004; Ritchie, 2006; Stocker, 2000). These attenuated *Salmonella* vaccines have also recently been used as delivery vectors for viral and other foreign antigens (Garmory *et al.,* 2005; Galen *et al*., 2009; Kwon *et al*., 2007; Loessner *et al*., 2008). Viral antigens that were successfully expressed in attenuated recombinant *Salmonella* vaccines include the human papillomavirus (HPV)-16 L1 protein (Benyacoub *et al*., 1999), Hepatitis B virus nucleocapsid (Schodel *et al.,* 1997), lymphocytic choriomeningitis virus (LCMV) nucleoprotein (Shams *et al*., 2001), feline immunodeficiency virus Gag (Tijhaar *et al*., 1997), rotavirus VP2 and VP6 antigens (Coste *et al.,* 2001), G glycoprotein from human

respiratory syncytial virus I (Shata *et al.*, 2001), SIV p27 capsid (Steger *et al*., 1999), HIV-1 gp120 antigen (Fouts *et al.*, 1995), and HIV-1 subtype C Gag (Chin'ombe *et al*., 2009b). These studies showed that vaccination with *Salmonella* vaccines expressing the above mentioned proteins was associated with good humoral (IgA and IgG) and cell-mediated (CD8+ T) responses in animal models.

To express a viral antigen in recombinant *Salmonella*, the coding DNA sequence of the gene is cloned into a prokaryotic expression plasmid vector. The expression plasmid contains the necessary transcription and translation domains such as the promoter, origin of replication, selection antibiotic resistance gene, Shine-Dalgarno (ribosome-binding site), transcription start and termination signals which are important for the expression of the foreign antigen (Hannig and Makrides, 1998; Jana and Deb, 2005). The recombinant expression plasmid is used in the transformation of the bacterium (Sambrook *et al.*, 1989). In previous studies, it was shown that the coding sequences of green fluorescent protein (GFP) and human immunodeficiency virus type 1 subtype C Gag could be expressed at high levels in attenuated *Salmonella enterica* serovar Typhimurium (Chin'ombe, 2007; Chin'ombe *et al*., 2009a,b; Seleem *et al*., 2008). In these studies, the genes of the foreign antigens were cloned into an expression plasmid and the expression was constitutively driven by the *E. coli* lactose (*lac*) promoter in pGEM®-Teasy plasmid from Promega Corporation (USA). The genes were fused in-frame with the *LacZα* gene in the plasmid (Chin'ombe, 2007; Chin'ombe *et al.*, 2009a,b). Mice vaccinated with the recombinant *Salmonella* bacteria elicited GFP and Gag-specific immune responses (Chin'ombe, 2007; Chin'ombe *et al*., 2009a,b). This showed that the recombinant bacterium successfully delivered the expressed foreign antigens to the immune system. It would, therefore, be interesting to use the same expression plasmid system to express HIV-1 subtype C Tat, Nef and reverse transcriptase in *Salmonella enterica* serovar Typhimurium.

4.2 OBJECTIVES

The first objective of this study was to develop recombinant *Salmonella enterica* serovar Typhimurium expressing Tat, Nef and reverse transcriptase. This was achieved by cloning the coding sequences of human immunodeficiency virus type 1

subtype C *tat*, *nef* and *reverse transcriptase* genes, individually, into the prokaryotic expression pGEM®-Teasy plasmid. The genes were to be fused in-frame with the *LacZa* gene and expression driven by the *E. coli lac* promoter. An attenuated mutant strain of *Salmonella enterica* serovar Typhimurium was transformed with the recombinant expression plasmids (pGEM+Tat, pGEM+Nef and pGEM+RT) harbouring the HIV-1 *tat*, *nef* and *rt* genes. This was done for extraction of HIV-1 subtype C Tat, Nef and RT proteins from *Salmonella enterica* serovar Typhimurium. The expression of the foreign antigens (Tat, Nef and RT) was evaluated by SDS-PAGE and Western blotting.

4.3 MATERIALS

The source and properties of the bacterial strains, *E. coli DH5α* cells, Δ*aro*C *Salmonella enterica* serovar Typhimurium mutant, and plasmids used in this study are given in Table 2.1. Restriction enzymes were supplied by Roche Diagnostics (Germany) and New England Biolabs (USA). Solutions and buffers are given in the Appendix A.

Table 2.1: The source and properties of bacterial strains and plasmids used in the study.

Material	Nature of material	Important properties	Source
DH5α	*E. coli* bacterium	For use in molecular manipulations	Stratagene, USA
Δ*aro*C	*Salmonella* bacterium	Mutated in the *aroC* gene	Dr N. Chin'ombe
pGEM+SalmgagGFP	plasmid	Contains the *gag-gfp (green fluorescence protein)* gene fused to the *lacZa* and expression under the *lac* promoter	Dr N. Chin'ombe
pScript+Tat	plasmid	Contains HIV-1 subtype C *tat* gene	Dr N. Chin'ombe
pScript+Nef	Plasmid	Contains HIV-1 subtype C *nef* gene	Dr N. Chin'ombe
pScript+RT	plasmid	Contains HIV-1 subtype C *rt* gene	Dr N. Chin'ombe

4.4 METHODS

A flow diagram of construction and evaluation of Salmonella *enterica* serovar Typhimurium expressing HIV-1 antigens (Tat, Nef and RT), is given in Figure 2.1). Briefly, HIV-1 genes (*tat*, *nef* and *rt*) were cloned from pScript+Tat, pScript+Nef and pScript+RT into a prokaryotic expression plasmid based on pGEM-Teasy vector and recombinant expression plasmids (designated pGEM+Tat, pGEM+Nef and pGEM+RT) were generated. The expression plasmids were used to transform the *Salmonella enterica* serovar Typhimurium. Evaluation of the expression of the HIV-1 Tat, Nef and RT proteins by the bacteria was done by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

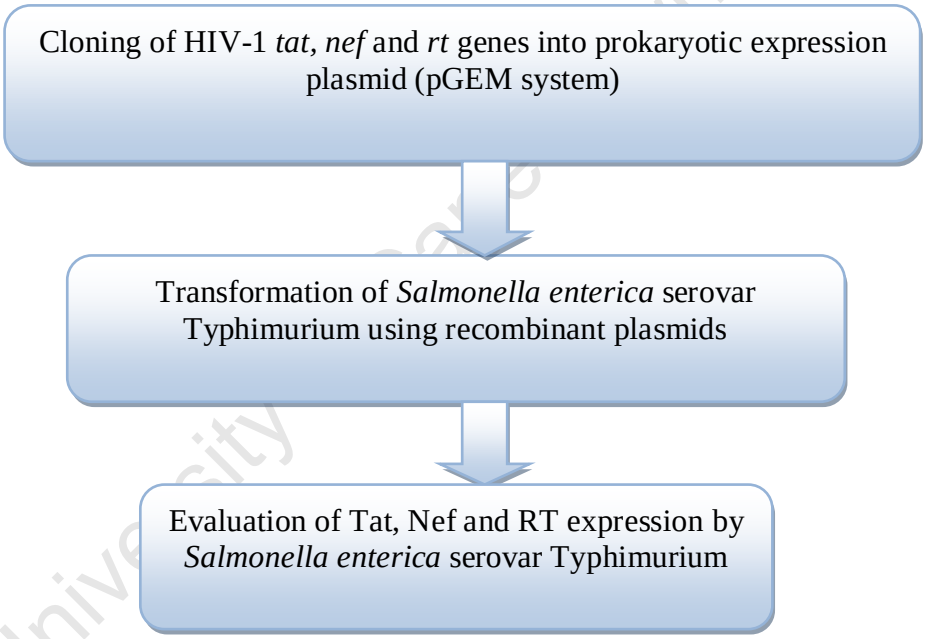

Figure 2.1 A flow Diagram of experimental strategy employed in chapter 2.

4.4.1 Restriction mapping of HIV-1 genes (*tat, nef* and *rt*) in pScript+Tat, pScript+Nef and pScript+RT

To check whether the provided plasmids (pScript+Tat, pScript+Nef and pScript+RT) contained the HIV-1 *tat*, *nef* and *rt* genes, restriction endonuclease mapping was used. The pScript+Tat, pScript+Nef and pScript+RT plasmids were digested using NarI (New England Biolabs, USA) and HpaI (Roche Diagnostics, Germany). The digestion contained: 500 ng of plasmid DNA, 1X restriction buffer (Roche Diagnostics, Germany), 5 U of enzyme and the reaction was done in a 20 µl volume.

The digestions were incubated at 37°C for 1 hr. After the incubation, 2 µl of 6X loading dye was added and the digestions were analysed by 1.5% gel (Appendix A: 11) electrophoresis.

4.4.2 Preparation of HIV-1 *tat, nef* and *rt* genes from pScript+Tat, pScript+Nef and pScript+RT for cloning

The *tat, nef,* and *rt* genes were excised out of the pScript+Tat, pScript+Nef and pScript+RT respectively using NarI and HpaI double digestion. Each digestion was prepared in a total volume of 50 µl with the following components: 1500 ng of plasmid DNA, 50 U of each enzyme and 1X of restriction buffer (Roche Diagnostics, Germany). Water was used to top-up the reaction volume to 50 µl. The digestions were incubated at 37°C for 2 hrs. The digestions were then loaded onto 1.2% agarose gel (2 wells/reaction) and run until the gene fragments were separated from the pScript plasmid backbone.

The *tat, nef* and *rt* bands were then excised from the agarose gel and purified using QIAGEN gel extraction kit according to manufacturer's instructions (QIAGEN, Germany). Briefly, the DNA fragments were excised from agarose gel, dissolved in Buffer QG (provided with the kit) and precipitated with isopropanol. The mixture was then transferred into a collection tube which was then put inside the provided QIAquick spin column and centrifuged at 10000 rpm (Eppendorf 5417C Centrifuge, Germany) for 1 min. The flow-through was discarded and 0.5 ml Buffer QG (reagent provided with the kit) was added to the collection tube and centrifuged again for 1 min. The pellet bound to the QIAquick column was washed with 0.75 ml of Buffer PE and the DNA was eluted with 30 µl of water by centrifuging again at 10000 rpm (Eppendorf 5417C Centrifuge, Germany) for 1 min.

The purified products were quantified by Nanodrop ND-1000 UV/VIS spectrophotometer (Thermo scientific, USA) with absorbance of the DNA read at a wavelength of 260nm (A_{260}) and 280nm (A_{280}) to evaluate the purity of the preparation. The presence of purified products were further analysed by 1.2% agarose gel electrophoresis and the expected sizes of *tat, nef* and *rt* genes were 429 bp, 655 bp and 1381 bp, respectively.

4.4.3 Preparation of plasmid expression (pGEM backbone) for cloning of *tat, nef* and *rt* genes

The plasmid expression (pGEM) backbone was prepared from pGEM+SalmgagGFP plasmid, which contained the HIV-1 *gag*-green fluorescence protein (*gfp*) fusion gene between NarI and HindIII. *Gag* and *gfp* genes were in-frame with 5' domain of *β*-galactosidase α-gene (*lacZa)*. The pGEM backbone was prepared by excising out the *gag* and *gfp* genes from the pGEM+SalmgagGFP plasmid. In order to remove the *gfp* fragment, the pGEM+SalmgagGFP was digested with HindIII in a total volume of 50 µl. The digestion contained 1500 ng plasmid DNA and 50 U of the enzyme. After 1 hr incubation, at 37°C, 10 U of *pfu* polymerase was added to blunt the HindIII-digested ends and incubated for another hour at 72°C. The digestion product was purified using QIAGEN gel extraction kit according to manufacturer's instructions (QIAGEN, Germany). In order to excise the *gag* fragment from the HindIII-digested pGEM+Salmgag plasmid backbone, the following components were added into the digestion mix: 10 µl of HindIII-digested pGEM+Salmgag product, 5 µl of 10X restriction buffer, 5 µl (50 U) of NarI enzyme and 30 µl of distilled water. The mix was incubated at 37°C for 2 hrs and then loaded on 1.2% agarose gel. When the DNA fragments had separated, the pGEM backbone was excised from the agarose gel and purified using the QIAGEN gel extraction kit according to manufacturer's instructions as previously described on section 2.4.2 (QIAGEN, Germany). The plasmid backbone was analyzed by 1.2% agarose gel electrophoresis DNA concentration determined by the Nanodrop ND-1000 UV/VIS spectrophotometer (Thermo scientific, USA).

4.4.4 Determination of DNA concentration by Nanodrop

The DNA of purified pGEM backbone, *tat, nef* and *rt* genes was quantified using a Nanodrop ND-1000 UV/VIS spectrophotometer (Thermo scientific, USA). The absorbance of the DNA was read at a wavelength of 260nm (A_{260}) and 280nm (A_{280}). The ratio between these wavelengths provides an approximate purity of the sample which is between 1.8 to 2.0 values.

2.4.5 Ligation of HIV-1 genes (*tat, nef* and *rt*) into pGEM backbone

The HIV-1 *tat, nef and rt* gene fragments, which were previously prepared (Section 2.4.2) were individually ligated into the previously-prepared pGEM plasmid backbone (Section 2.4.3) as shown on Figures 2.2-2.4. Each ligation reaction contained vector DNA (80 ng) and insert DNA (40 ng), 1X ligation buffer (Roche, Germany), 20 U of T4 DNA ligase (Roche Diagnostics, Germany) and the reaction was done in a total volume of 10 µl. Ligation digestions were prepared on ice and incubated for overnight at 4°C. Two ligation controls were included, (1) a negative ligation control including a ligation digestion with only vector but no insert. This was used to identify if there were any false results due to the re-ligation of the vector, (2) pScript+Tat plasmid DNA was used as positive control to identify the positive results. The products of the ligation digestion were ready for transformation into *E. coli DH5α* cells.

2.4.6 Transformation of competent *E. coli DH5α* cells with plasmid

Competent *E. coli DH5α* bacterial cells were prepared using the calcium chloride method (Sambrook *et al.,* 1989). Briefly, 100 µl of culture from *E. coli* stock were streaked onto 2x yeast tryptone (YT) agar plates (Appendix A: 1) and grown at 37^{o}C overnight. A single colony was selected from the plate and inoculated in a total volume of 15 ml of 2xYT media (Appendix A: 2) and grown at 37^{o}C overnight shaking. A 5 ml of the culture was diluted into 200 ml of 2xYT media (Appendix A: 2) and grown until the cells reached logarithmic phase (Absorbance values between 0.5 and 0.6 at 600 nm). The bacterial cultures (100 ml) were harvested at 5000 rpm (Sorvall RC-5C Plus superspeed centrifuge, Rotor S/N 10300651, USA) for 5 min at 4^{o}C and suspended in 0.1 M ice-cold calcium chloride (Appendix A: 8). The cells were incubated on ice for 60 min. The cells were reharvested by centrifugation and resuspended in 8 ml of ice-cold 0.1 M calcium chloride containing 10% glycerol solution (Appendix A: 7). The cells were stored at -80°C in 100 µl aliquots.

The ligation reactions (section 2.2.4) were used to transform the competent bacterial cells using the heat-shock method (Sambrook *et al.,* 1989). Briefly, frozen 100 µl aliquots of competent *E. coli DH5α* cells were thawed on ice and 10 µl of each ligation was added. The cells and the ligations were mixed gently and incubated on

ice for 30 min. The cells were heat-shocked at 42°C in a water bath for 2 min and incubated on ice for 2 min. Nine-hundred microliters (µl) of 2xYT broth was added and the cells were incubated at 37°C for 1 hr. The cells were then plated onto 2xYT agar supplemented with 100 µg/ml ampicillin (Appendix A: 3) and incubated at 37°C overnight. The following morning, the bacterial colonies from each transformation were counted.

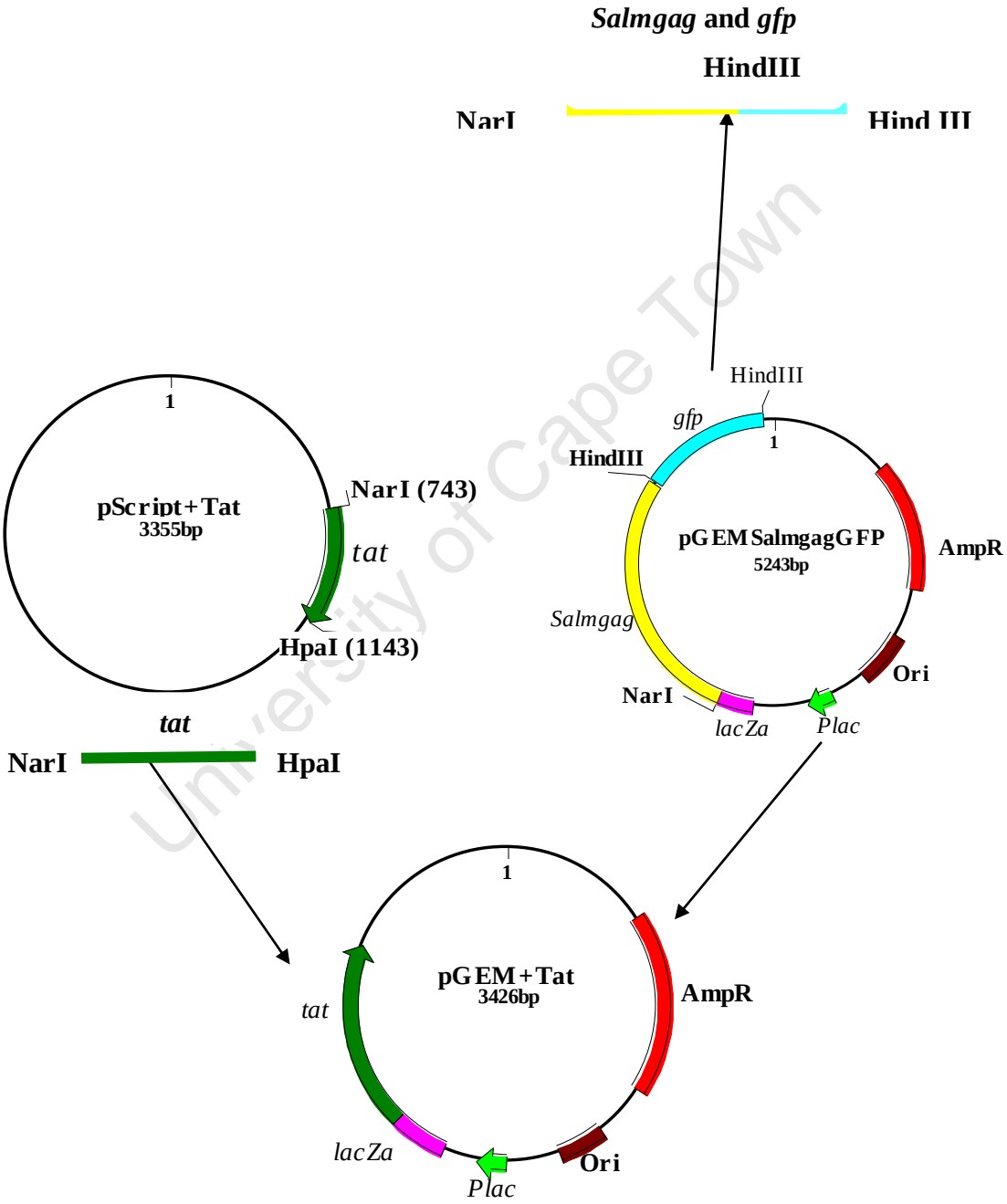

Figure 2.2 **Construction of pGEM+Tat by DNAMAN software version 4.0 (Lynnon Biosoft, Canada).** The *tat* gene was digested from pScript+Tat using NarI (sticky cutter) and HpaI (blunt cutter). The gene was cloned into the pGEM backbone prepared from digesting pGEM+SalmgagGFP by excision with of HIV-1 *gag and gfp*.

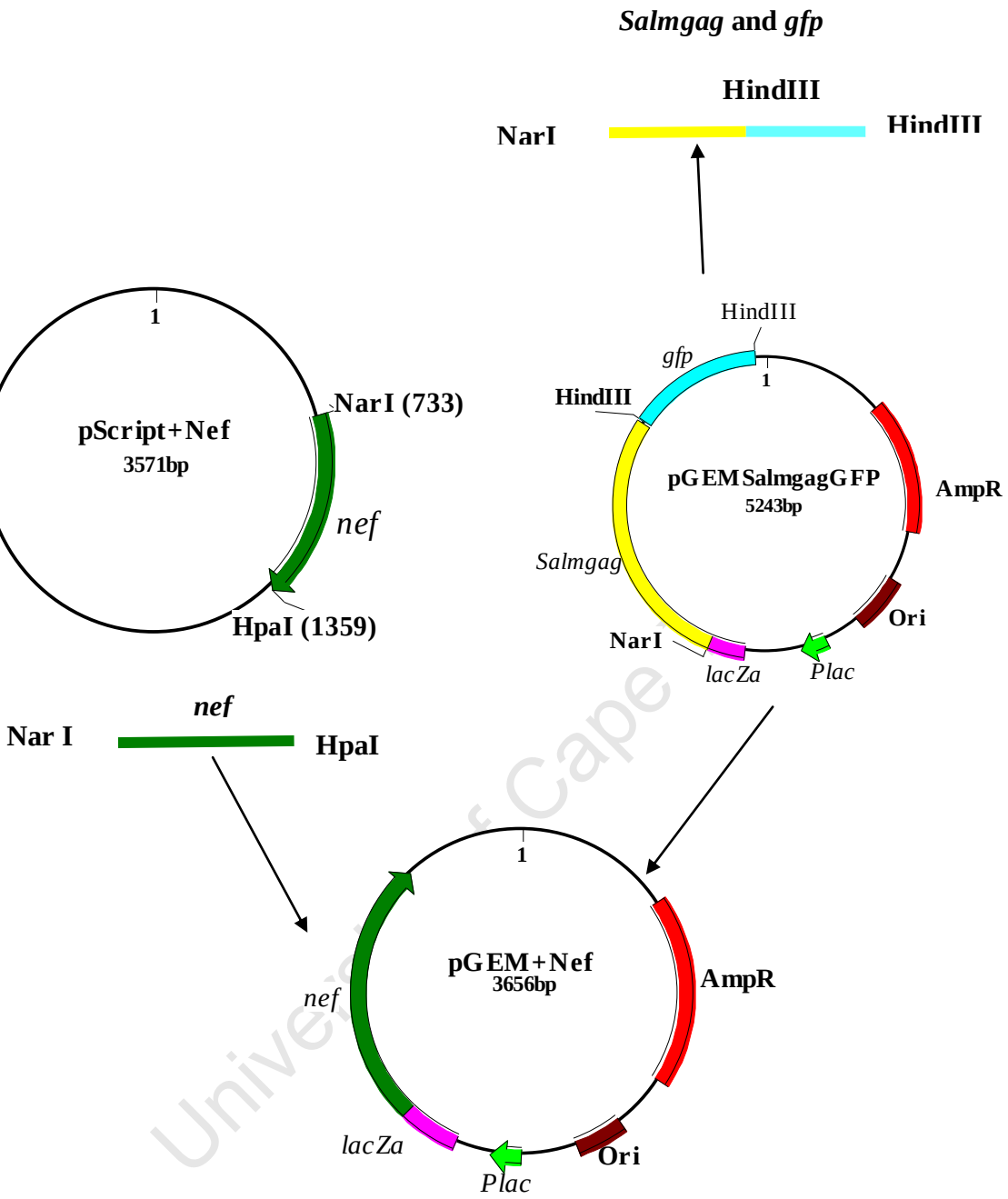

Figure 2.3: Construction of pGEM+Nef by DNAMAN software version 4.0 (Lynnon Biosoft, Canada). The *nef* gene was digested from pScript+Nef using NarI (sticky cutter) and HpaI (blunt cutter). The gene was cloned into the pGEM previously prepared from pGEM+SalmgagGFP.

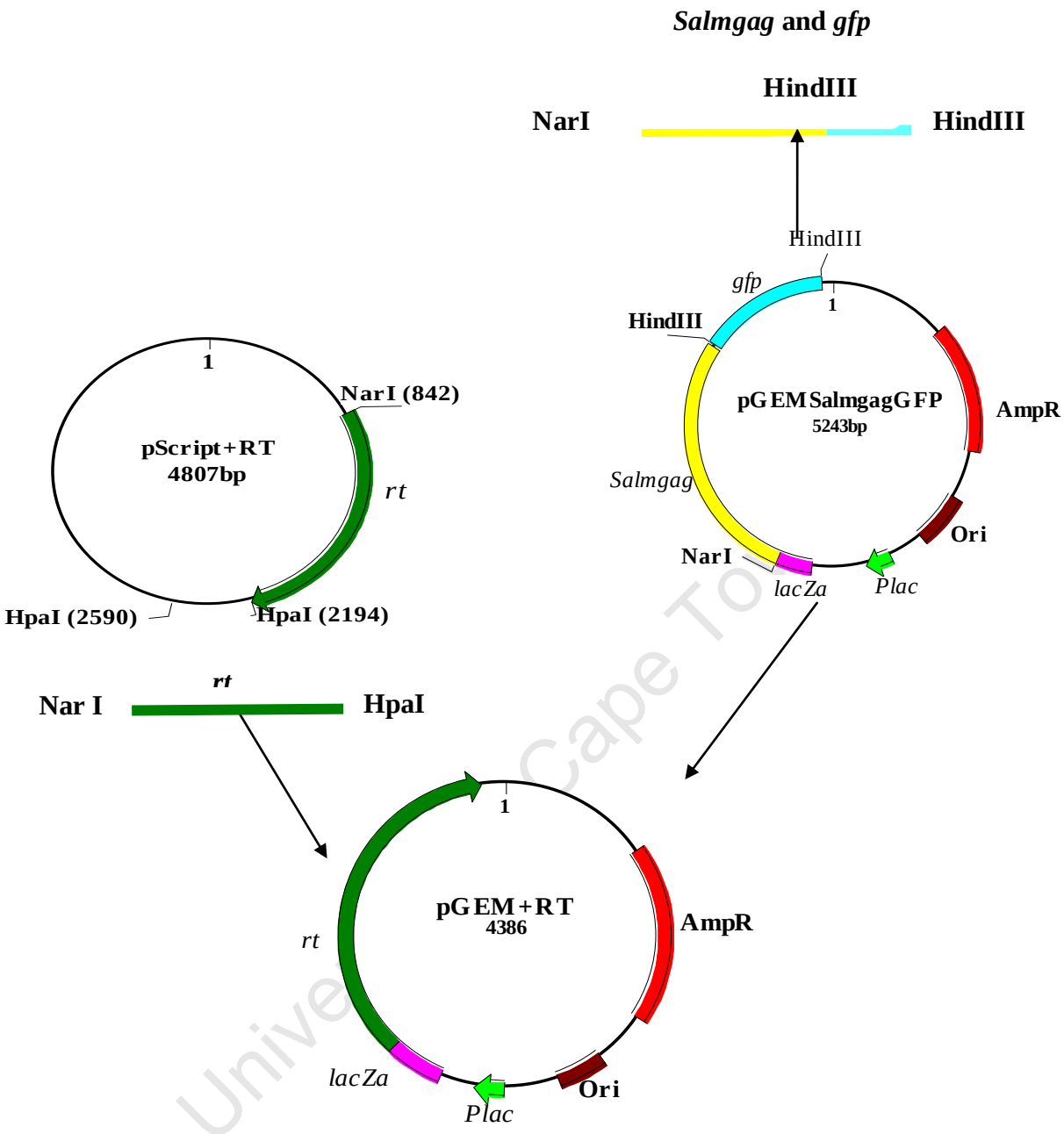

Figure 2.4: Construction of pGEM+RT by DNAMAN software version 4.0 (Lynnon Biosoft, Canada). The *rt* gene was digested from pScript+RT using NarI (sticky cutter) and HpaI (blunt cutter). The gene was cloned into the pGEM previously prepared from pGEM+SalmgagGFP.

2.4.7 Screening of recombinant *E. coli DH5α* colonies for correct plasmid

Bacterial colonies (5 per plate) from each transformation were inoculated into 15 ml 2xYT media supplemented with 100 µg/ml ampicillin (Appendix A: 3) and incubated at 37°C overnight. The following morning, plasmid DNA was isolated using an alkaline/SDS method (Sambrook *et al.*, 1989). Briefly, 4 ml of each

bacterial culture was harvested by centrifugation at 5000 rpm (Eppendorf 5417C Centrifuge, Germany) for 5 min and the pellet was resuspended in 400 µl of resuspension buffer (Appendix A: 12). Freshly prepared lysis buffer (400 µl) (Appendix A: 13) was added to the cells and the suspension was incubated at room temperature for 5 min. A neutralization buffer (400 µl) (Appendix A: 14) was added and the suspension was inverted 4-6 times before incubation on ice for 5 min. After pelleting cell debris at 14000 rpm (Eppendorf 5417C Centrifuge, Germany) for 10 min, 900 µl of the supernatant was added to 644 µl of isopropanol in a new eppendorf tube and centrifuged at 14000 rpm (Eppendorf 5417C Centrifuge, Germany) for 15 min. The pelleted plasmid DNA was washed with 700 µl of 70% ethanol (Appendix A: 15) by centrifuging at 14000 rpm (Eppendorf 5417C Centrifuge, Germany) for 10 min. The plasmid DNA pellet from each preparation was dried and suspended in 30 µl of distilled water. The quantities of the DNA preparations were done using the Nanodrop ND-1000 UV/VIS spectrophotometer (Thermo scientific, USA) as described previously (section 2.4.4).

The recombinant plasmids (designated pGEM+Tat, pGEM+Nef and pGEM+RT) were screened by restriction enzyme mapping using EcoRI (Roche Diagnostics, Germany) that cut at the N-terminus and C-terminus of each gene (*tat*, *nef* and *rt*, respectively). The restriction digestion of each plasmid contained 500 ng of plasmid DNA, 1X of restriction buffer and 10 U of restriction enzyme. The digestion was made in a total volume of 20 µl at 37°C for 2 hrs. The digested DNA was analysed using 1.5% agarose gel electrophoresis. To confirm the presence of the *tat*, *nef* and *rt* genes in selected pGEM+Tat, pGEM+Nef and pGEM+RT, respectively, the plasmids were sent to Central DNA Sequencing Facility for sequencing (Department of Genetics, University of Stellenbosch). The sample reactions included 100 ng of plasmid DNA (pGEM+Tat, pGEM+Nef and pGEM+RT), 1 pmol/µl of M13 forward sequencing primer {5'-(CGCCAGGGTTTTCCCAGTCACGAC)-3'} and 1 pmol/µl M13 reverse sequencing primer {5´-(TCACACAGGAAACAGCTATGAC)-3´}. The product sizes of pGEM+Tat, pGEM+Nef and pGEM+RT plasmid were 3426 bp, 3652 bp and 4378 bp, respectively. The DNA sequences for each plasmid were analysed using DNAMAN software version 4.0 (Lynnon Biosoft, Canada).

2.4.8 Transformation of *Salmonella enterica* serovar Typhimurium

The growth conditions for *Salmonella enterica* serovar Typhimurium and *E. coli* were the same except that 100 µg/ml aromix compounds (Appendix A: 5) and 100 µg/ml tyrosine (Appendix A: 6) were added into media for *Salmonella*. Competent *Salmonella enterica* serovar Typhimurium cells were prepared using calcium chloride as previously described for *E. coli* (section 2.4.6). The cells were transformed with the pGEM+Tat, pGEM+Nef and pGEM+RT plasmids using the heat-shock method as previously described. The plates were incubated overnight at 37°C.

2.4.9 Extraction of total bacterial protein from *Salmonella enterica* serovar Typhimurium

A single bacterial colony from recombinant *Salmonella enterica* serovar Typhimurium harbouring the pGEM+Tat, pGEM+Nef or pGEM+RT was inoculated into 100 ml 2xYT media with 100 µg/ml of ampicillin, aromix and tyrosine (Appendix A: 3, 5 and 6) and grown at 37^{o}C overnight on a shaker. Extraction of the total protein from recombinant *Salmonella enterica* serovar Typhimurium was performed using the SDS-lysis method. The bacterial culture (4 ml) was pelleted at 6000 rpm (Eppendorf 5417C Centrifuge, Germany) for 5 min at 4^{o}C. The pellet was washed with 1ml of cold PBS (pH 7.4) at 14000 rpm (Eppendorf 5417C Centrifuge, Germany) for 10 min at 4^{o}C and resuspended in 500 µl of protein lysis buffer (Appendix A: 26). The suspension was boiled in a water bath for 30 min and the supernatant containing the total protein was clarified by centrifuging at 14000 rpm (Eppendorf 5417C Centrifuge, Germany) for 10 min at 4^{o}C. The supernatant was transferred into a new Eppendorf tube and the protein concentrations were quantified.

2.4.10 Determination of protein concentration by Bio-Rad DC assay

The concentration of the extracted recombinant *Salmonella* protein was determined using the Bio-Rad DC protein assay kit according to manufacturer's instructions. The bovine serum albumin (BSA) was used to prepare a standard curve. Several dilutions (0.1, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 µg/µl) of BSA were made with 1X CAT lysis buffer and the absorbance was measured at 750nm using a VERSmax microplate reader (Bio-rad, Canada). Using the absorbance measured, the BSA standard curve was made and used to determine the concentration of protein samples in µg/µl.

2.4.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

In order to confirm the protein expression by recombinant bacteria, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. A 12.5% resolving gel was prepared (Appendix A: 22) and poured into the plates leaving approximately 4 cm on top of the resolving gel for the stacking gel. The resolving gel was overlaid with water-saturated butanol and allowed to solidify at room temperature for about an hour. After the gel was polymerised, the butanol was removed. A 4% stacking gel was prepared (Appendix A: 23) and poured into the space left in the plates on top of the resolving gel. The sample comb was inserted into 4% stacking gel to generate sample wells and allowed it to polymerise at room temperature for an about 30 min. Once the gel was polymerized, 20 µl of 500 ng protein was mixed with 2X Laemmli loading buffer (Appendix A: 21) at a ratio of 1:4 and boiled for 5 min to denature the proteins. The protein samples (20 µl per well) were loaded and run for 15 min at 60 V and a further 2 hrs at 80 V. A spectrum broad range colour stained marker (Inqaba biotec, Canada) was loaded to identify the sizes of the proteins as well as to monitor the protein separation in the gel during electrophoresis. Two duplicate SDS-PAGE gels were always prepared: one gel for Coomassie blue staining (Appendix A: 34) and the other gel for Western blotting.

2.4.12 Coomassie blue staining and destaining

Coomassie Brilliant blue solution (Appendix A: 34) was used to stain the SDS-PAGE gels. The gels were covered with Coomassie blue staining solution and incubated on a slight shaking for about 2 hrs. To destain the protein gels, the staining was removed and washed with destaining solution (Appendix A: 35) 5 times on a shaker (Orbital Shaker $SO_{3,}$ USA) and left on destaining solution overnight at room temperature. The destained gel was then placed on a light box and photographed with a Sony DSC-P73 digital camera.

2.4.13 Western blotting of total bacterial proteins to detect recombinant proteins

Total bacterial proteins (only from *Salmonella* expressing Nef or RT) were transferred from the SDS-PAGE gel to the Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham, UK) by Western blotting. The SDS-PAGE gel was equilibrated in 1X transfer buffer (Appendix A: 33) for 20 min. While waiting for equilibration of the electrophoresis gel, the Hybond-P PVDF membrane was cut to

the dimensions of the gel and pre-wetted in 100% methanol for 5 min, rinsed with distilled water for 10 min and equilibrated in the transfer buffer for 20 min. The filter papers were also cut to the dimensions of the gel and saturated by soaking in the transfer buffer for 20 min. The saturated filter paper was placed bottom, followed by membrane and a gel on top of the membrane. The other pre-soaked filter paper was placed on top of the gel. Each layer of the sandwich was rolled over with a glass pipette to remove the air bubbles. Electroblotting was carried out by applying a constant voltage of 15 V for 60 min at room temperature. The membrane was incubated in blocking buffer (Appendix A: 30) at 4^{o}C overnight prior to immunodetection.

2.4.14 Immunodetection of recombinant Nef and RT on Western blots

To detect Nef or RT from the Western blots, mouse anti-Nef (Sigma, Germany) and rabbit anti-RT (Sigma, Germany) antibodies respectively, diluted 1:500, were added after removal of blocking buffer. The blots were incubated for 2 hrs at room temperature and then washed four times with an excess amount of washing buffer (Appendix A: 30) for 10 min each. A secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Sigma, Germany) and a sheep anti-rabbit IgG antibody conjugated horseradish peroxidase (Sigma, Germany) (diluted 1.1000) were added to the respective blots. The blots were incubated for 1 hr at room temperature. The blots were washed four times with washing buffer (Appendix A: 30) for 10 min each. The Nef was detected from the blot using NBT-BCIP (Nitro Blue Tetrazolium chloride-5-Bromo-4-Chloro-3-Indolyl Phosphate) substrate (Roche Diagnostics, Germany) following the manufacturer's instructions. The RT protein was detected from the blot using the Nova Red Substrate solution (Vector Laboratories, CA, USA). The blots were covered with the substrate solutions until Nef or RT protein bands had developed. The blots were rinsed in water, air-dried and scanned (CannonScan 8000F, China).

2.5 RESULTS

2.5.1 Verification of the plasmids with restriction mapping

The pScript+Tat, pScript+Nef and pScript+RT plasmids containing the HIV-1 *tat*, *nef* and *rt* genes, respectively, were mapped using NarI and HpaI restriction enzymes. The generated DNA fragments were analysed by agarose gel electrophoresis and they were all observed with expected molecular weights (Figure 2.5 A-C, lanes 2). The sizes of the produced fragments are given in Table 2.1. The expected DNA sizes of *tat*, *nef* and *rt* fragments after restriction digestions were 400 bp, 626 bp and 1352 bp respectively (Table 2.1). The pScript+RT generated three bands (Figure 2.5C, lane 2), where the 510 bp was *p51* (*rt*) cloned between two HpaI sites and the 1352 bp was the other *rt* gene (*p66*) cloned between NarI and HpaI. The pScript backbone band was 2945 bp.

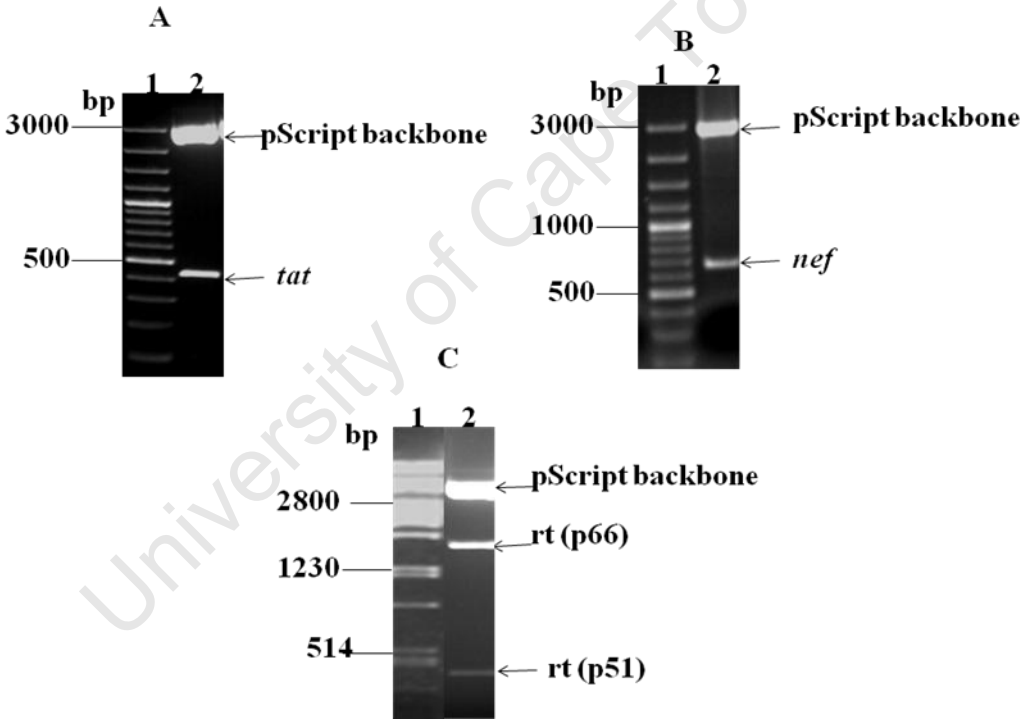

Figure 2.5: **Restriction mapping of pScript+Tat, pScript+Nef and pScript+RT plasmids using NarI and HpaI.** (**A**) **Restriction mapping of pScript+Tat**. **Lane 1:** O'Generuler 100 bp DNA ladder, **Lane 2**: pScript+Tat digested with NarI and HpaI, (**B**) **Restriction mapping of pScript+Nef.** Lane 1: O'Generuler 100 bp DNA ladder, **Lane 2:** pScript+Nef digested with NarI and HpaI. (**C**) **Restriction mapping of pScript+RT**. **Lane 1**: λDNA/PstI molecular weight marker. **Lane 2**: pScript+RT digested with NarI and HpaI.

Table 2.1: Predicted sizes of DNA after restriction mapping of pScript+Tat, pScript+Nef and pScript+RT

Plasmid Name	Restriction Enzymes	Expected DNA band sizes (bp)
pScript+Tat	NarI and HpaI	400 and 2945
pScript+Nef	NarI and HpaI	626 and 2945
pScript+RT	NarI and HpaI	510, 1352 and 2945

2.5.2 HIV-1 *tat, nef* and *rt* cloning into pGEM expression plasmid backbone

The pGEM+SalmgagGFP had *gag* and *green fluorescence protein* (*gfp*) genes cloned in-frame with 5' domain of *β*-galactosidase α-gene (*lacZa*). In order to remove *gag* and *gfp*, pGEM+SalmgagGFP was initially digested with HindIII to release the *gfp* gene (Figure 2.6A). Two DNA bands were expected on the agarose gel (Figure 2.6A; Table 2.2). The smaller DNA fragment was the *gfp* gene and the bigger fragment was the linearized plasmid which still contained the HIV-1 *gag* gene. The ends of the plasmid were then blunt-ended using *pfu* polymerase and the plasmid was further digested with NarI to excise out the HIV-1 *gag* gene (Figure 2.6B). The released HIV-1 *gag* gene was 1470 bp and the pGEM plasmid backbone was 3026 bp (Figure 26B, Table 2.2). There was incomplete digestion of the plasmid with NarI and there were other bands above the 3026 bp band (Figure 2.6B, lane 2). A 3026 bp band for the pGEM plasmid backbone was cut and gel-purified (Figure 2.6C, lane 2).

The HIV-1 *tat*, *nef* and *rt* genes were excised from the pScript+Tat, pScript+Nef and pScript+RT plasmids respectively using NarI and HpaI and purified (Figure 2.7). The expected sizes of the genes were observed on agarose gels (Figure 2.7; A, lane 2; B, lane 2 & C, lane 2). In order to construct the pGEM+Tat, pGEM+Nef and pGEM+RT plasmids, the *tat*, *nef* and *rt* genes were individually ligated into the pGEM backbone and competent *E. coli* cells transformed. Most colonies screened had plasmid DNA which contained the respective HIV-1 (*tat*, *nef* and *rt*) genes (data not shown). The restriction mapping of the candidate plasmids DNA containing pGEM+Tat, pGEM+Nef and pGEM+RT, respectively, was performed using EcoR1

(Figure 2.8; A, lane 2; B, lane 2 & C, lanes 2). The frame of the HIV-1 *tat*, *nef* and *rt* genes in pGEM+Tat, pGEM+Nef and pGEM+RT was further confirmed by sequencing the plasmids (section 2.5.3).

Table 2.2: Predicted sizes of DNA after restriction digestion of pGEM+SalmgagGFP to prepare pGEM backbone

Plasmid	Restriction Enzymes	Expected DNA band sizes (bp)
pGEM+SalmgagGFP	HindIII	747 and 4496
Linearized pGEM+Salmgag	HindIII and NarI	747, 1470 and 3026

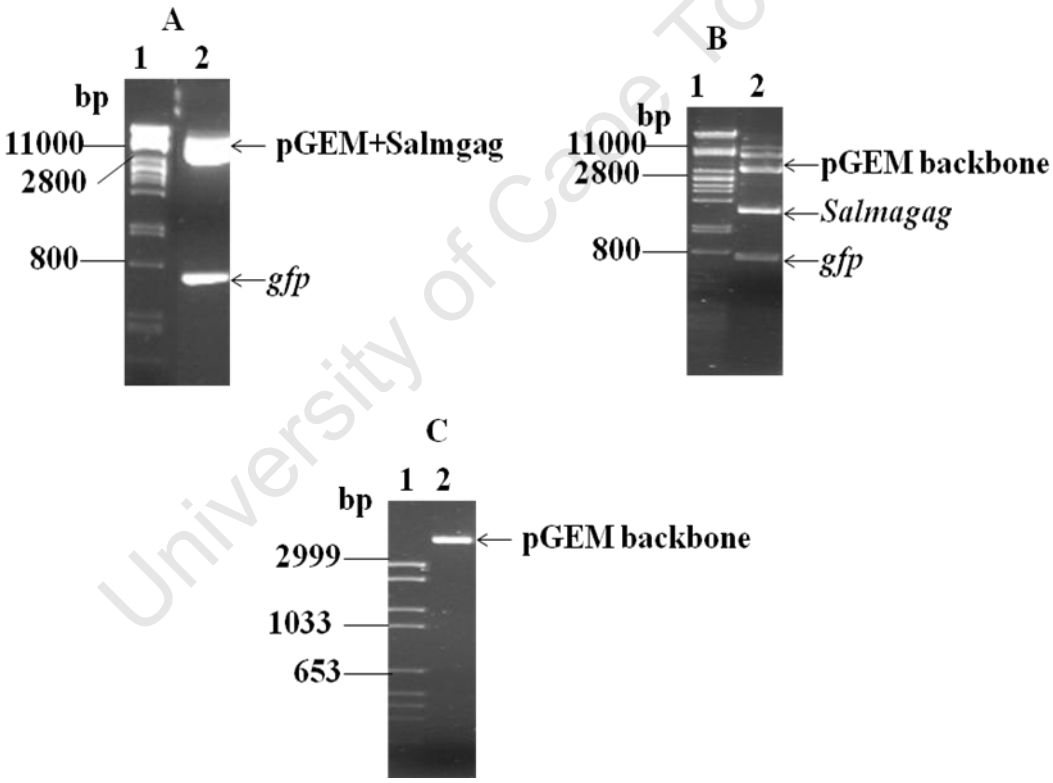

Figure 2.6: **Preparation of pGEM backbone. (A)** The pGEM+SalmgagGFP plasmid was digested with HindIII and blunt-ended with *Pfu* polymerase. **Lane 1**: λDNA/PstI DNA ladder, **Lane 2**: Linearized pGEM+Salmgag (upper band) and *gfp* (lower band) after digestion with HindIII **(B)** Linearized pGEM+Salmgag after digestion with NarI to release gag fragment. **Lane 1**: λDNA/PstI DNA ladder, **Lane 2**: pGEM backbone (upper band), *gag* (middle band) and *gfp* (lower band) **(C)** Gel-purified pGEM backbone. **Lane 1**: Marker VI, **Lane 2**: pGEM backbone.

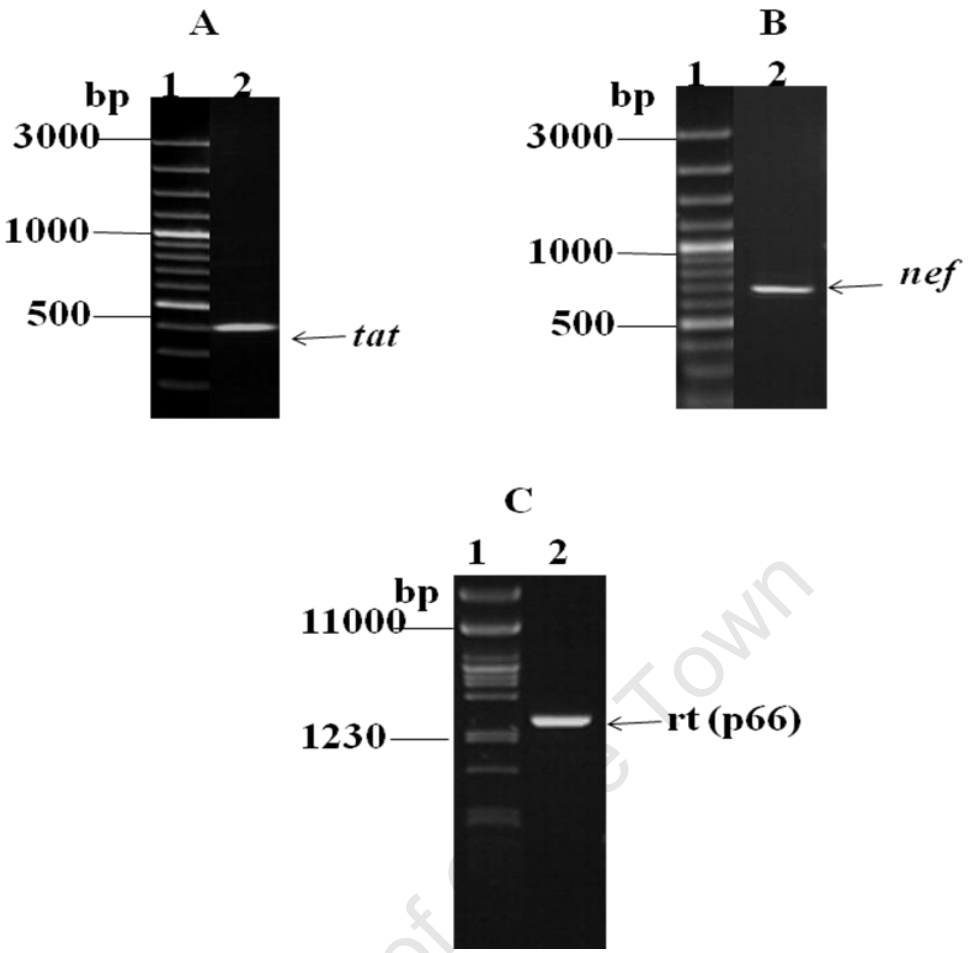

Figure 2.7: Purified HIV-1 *tat, nef* and *rt* genes. (**A**) A *tat* fragment was excised (Figure 2.6 A) and gel-purified. **Lane 1**: O'Generuler 100 bp DNA ladder, **Lane 2**: *tat* gene (**B**) A *nef* fragment was excised (Figure 2.6 B) and gel-purified. **Lane 1**: O'Generuler 100 bp DNA ladder, **Lane 2**: *nef* gene. (**C**) A *rt* fragment was excised (Figure 2.6 C) and gel-purified. **Lane 1**: O'Generuler 1kb DNA ladder, **Lane 2**: *rt* gene.

Table 2.3: Predicted sizes of DNA after restriction mapping of candidate pGEM+Tat, pGEM+Nef and pGEM+RT plasmids

Plasmid	Restriction Enzyme	Expected DNA band sizes (bp)
pGEM+Tat	EcoRI	429 and 2997
pGEM+Nef	EcoRI	655 and 2997
pGEM+RT	EcoRI	1381 and 2997

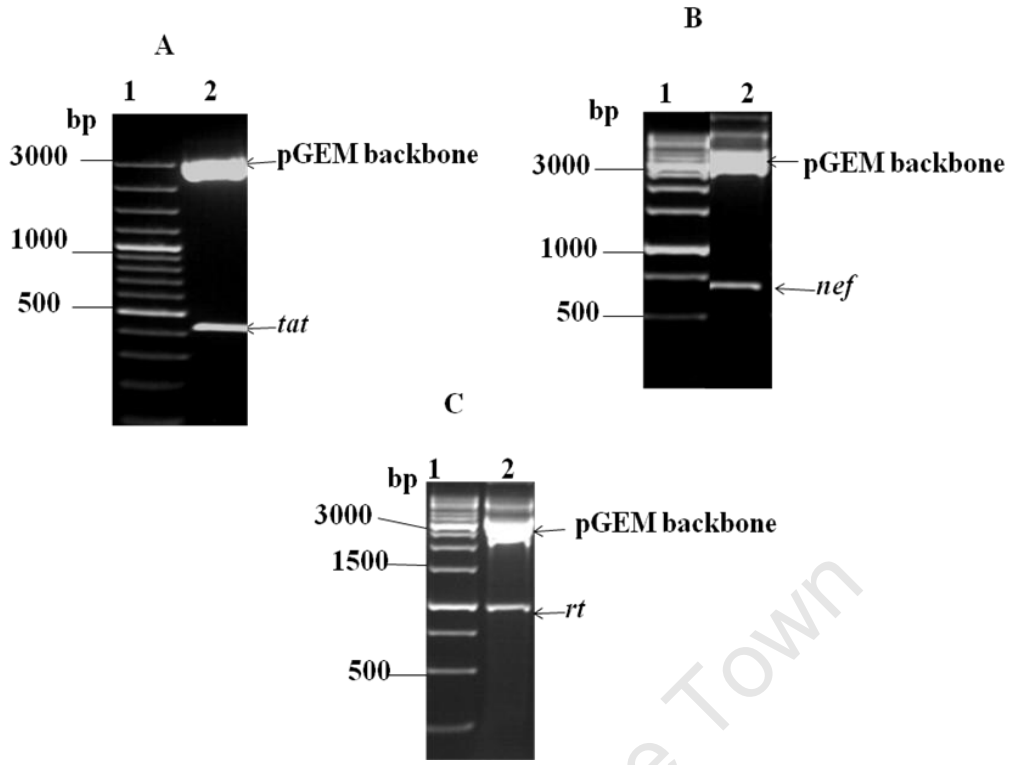

Figure 2.8: Restriction analysis of pGEM+Tat, pGEM+Nef and pGEM+RT. (**A**) A pGEM+Tat plasmid digested with EcoRI. **Lane 1**: O'Generuler 100 bp DNA ladder, **Lane 2**: pGEM+Tat. (**B**) A pGEM+Nef plasmid digested with EcoRI. **Lane 1**: O'Generuler 1kb DNA ladder, **Lane 2**: pGEM+Nef. (**C**) A pGEM+RT plasmid digested with EcoRI. **Lane 1**: O'Generuler 1 kb DNA ladder, **Lane 2**: pGEM+RT digested with EcoRI.

2.5.3 Sequencing of pGEM+Tat, pGEM+Nef and pGEM+RT expression plasmids

The candidate plasmids, pGEM+Tat, pGEM+Nef and pGEM+RT, were sequenced to confirm the restriction mapping results. The sequences were confirmed to be correct according to the *rt*, *tat and nef* gene sequences (Burgers *et al.,* 2006) using DNAMAN version 4.0 sequence analysis software (Lynnon Biosoft, Canada) {Figure 2.10A (*tat* gene from 30 bp to 504 bp), Figure 2.10B (*nef* gene from 30 bp to 729 bp) and Figure 2.10C (*rt* gene from 32 bp to `1467 bp)}. Based on the DNA sequence data, the maps of the three plasmids (pGEM+Tat, pGEM+Nef and pGEM+RT) were drawn (Figure 2.9, A - C). The DNA coding and protein sequences of the expressed proteins (Tat, Nef and RT) were also concluded from the sequence data (Figure 2.10, A - C). The pGEM+Tat, pGEM+Nef and pGEM+RT plasmids DNA had the *tat*, *nef* and *rt* genes, respectively, fused in-frame with *E. coli β*-galactosidase α-fragment (*lacZ*α) sequence under the expression of the upstream *E. coli lac* promoter. The expected band sizes of the *β*-galactosidase-Tat fusion protein,

β-galactosidase-Nef and β-galactosidase-RT fusion proteins were 19 kDa, 30 kDa and 59 kDa, respectively. The genes used the ATG start codon of β-galactosidase and they all had TAA as a stop codon.

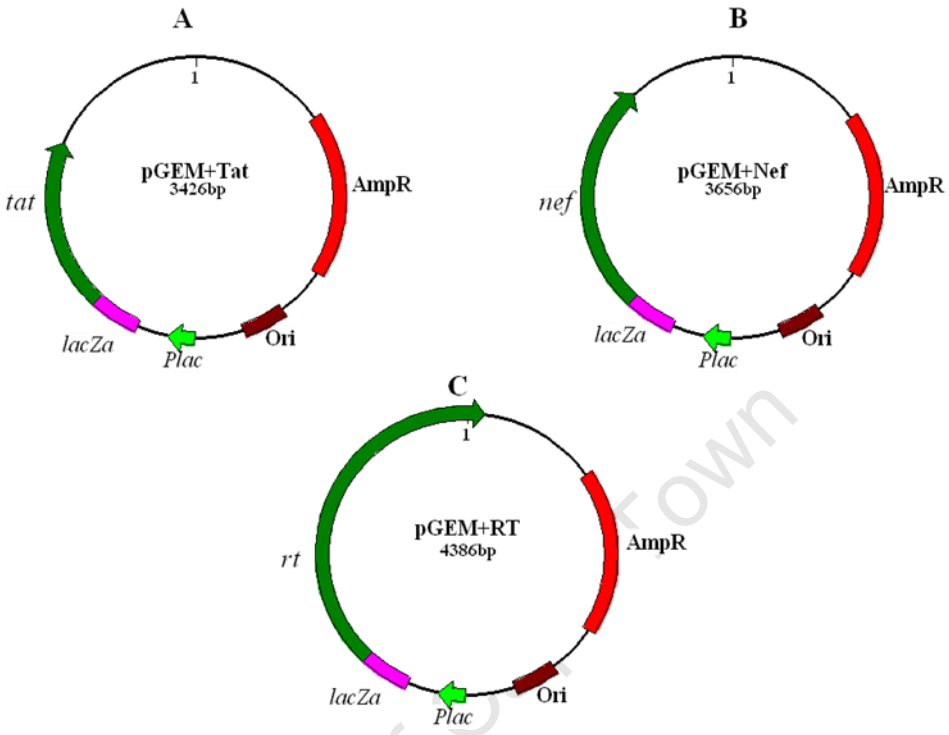

Figure 2.9: **Maps of pGEM+Tat, pGEM+Nef and pGEM+RT expression plasmids, constructed using DNAMAN software version 4.0 (Lynnon Biosoft, Canada).** These plasmids had HIV-1 subtype C *tat*, *nef* and *rt* genes which were previously cloned into the pGEM backbone fused in frame with the β-galactosidase α-gene (*lacZa*). The expression of the fusion protein was the under upstream *E. coli lac* promoter**.**

A

```
1          ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1            M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61         AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT
21           N  A  L  G  A  L  P  Y  G  R  P  A  G  G  R  E  F  T  S  D

121        TATGGCGCCCATATGCTGGGCATTAGCTATGGCCGCAAAAAACGTCGTCAGCGTCGTAGC
41           Y  G  A  H  M  L  G  I  S  Y  G  R  K  K  R  R  Q  R  R  S

181        ACCCCGCCGAGCAGCGAAGATCATCAGAACCCGATTAGCAAACAGCCGCTGCCGCAGACC
61           T  P  P  S  S  E  D  H  Q  N  P  I  S  K  Q  P  L  P  Q  T

241        CGTGGCGATCCGACCGGCAGCGAAGAAAGCAAAAAAAAAGTCGAAAGCAAAACCAAAACC
81           R  G  D  P  T  G  S  E  E  S  K  K  K  V  E  S  K  T  K  T

301        GATCCGTTTGATTGCAAATATTGCAGCTATCACTGCCTGGTGTGCTTTCAGACCAAAGGT
101          D  P  F  D  C  K  Y  C  S  Y  H  C  L  V  C  F  Q  T  K  G

361        CTGGGTATCTCCTACGGTCGTAAAAAGCGCATGGAACCGATTGATCCGAATCTGGAACCG
121          L  G  I  S  Y  G  R  K  K  R  M  E  P  I  D  P  N  L  E  P

421        TGGAATCATCCGGGCAGCCAGCCGAATACCCCGTGCAACAAATGCTATTGTAAGTACTGT
141          W  N  H  P  G  S  Q  P  N  T  P  C  N  K  C  Y  C  K  Y  C
```

```
481       TCCTACCATTGCTTGGTCGACTAA
161        S  Y  H  C  L  V  D  *
```

B

```
1         ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1          M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61        AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT
21         N  A  L  G  A  L  P  Y  G  R  P  A  G  G  R  E  F  T  S  D

121       TATGGCGCCCATATGGTGGGCTGGCCGGCGGTGCGTGAACGTATTCGTCGTACCGAACCG
41         Y  G  A  H  M  V  G  W  P  A  V  R  E  R  I  R  R  T  E  P

181       GCGGCGGAAGGCGTGGGCGCGGCGAGCCAGGATCTGGATAAACATGGCGCGCTGACCAGC
61         A  A  E  G  V  G  A  A  S  Q  D  L  D  K  H  G  A  L  T  S

241       AGCAATACCGCGCATAACAATCCGGATTGCGCGTGGCTGCAAGCGCAGGAAGAAGAACCG
81         S  N  T  A  H  N  N  P  D  C  A  W  L  Q  A  Q  E  E  E  P

301       GAAGTCGGCTTTCCGGTCCGTCCGCAGGTGCCGCTGCGTCCGATGACCTATAAAGCGGCG
101        E  V  G  F  P  V  R  P  Q  V  P  L  R  P  M  T  Y  K  A  A

361       TTTGATCTGAGCTTTTTTCTGAAAGAAAAAGGCGGCCTGGAGGGCCTGATCTATAGCAAA
121        F  D  L  S  F  F  L  K  E  K  G  G  L  E  G  L  I  Y  S  K

421       AAACGCCAGGATATTCTGGATTTATGGGTCTATCATACCCAGGGCTATTTTCCGGATTGG
141        K  R  Q  D  I  L  D  L  W  V  Y  H  T  Q  G  Y  F  P  D  W

481       CAGAATTATACCCCGGGTCCGGGCGTGCGTCTGCCGCTGACCTTTGGCTGGTGCTTTAAA
161        Q  N  Y  T  P  G  P  G  V  R  L  P  L  T  F  G  W  C  F  K

541       CTGGTGCCGGTCGATCCGGAAGAAGTGGAAGAAGCGAACAAAGGCGAAAACAACTGTCTG
181        L  V  P  V  D  P  E  E  V  E  E  A  N  K  G  E  N  N  C  L

601       CTGCATCCGCTGAGCCAGCATGGCATGGAAGATGCGGATCGCGAAGTCCTGAAATGGGTC
201        L  H  P  L  S  Q  H  G  M  E  D  A  D  R  E  V  L  K  W  V

661       TTTGATAGCAGCCTGGCGCGTCGTCATCTGGCGCGTGAAAAACATCCGGAATATTATAAA
221        F  D  S  S  L  A  R  R  H  L  A  R  E  K  H  P  E  Y  Y  K

721       GATGCATAA
241        D  A  *
```

C

```
1         ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1          M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61        AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT
21         N  A  L  G  A  L  P  Y  G  R  P  A  G  G  R  E  F  T  S  D

121       TATGGCGCCCATATGGGGCCCATTAGCCCGATTGAAACCGTGCCGGTGAAACTGAAACCG
41         Y  G  A  H  M  G  P  I  S  P  I  E  T  V  P  V  K  L  K  P

181       GGCATGGATGGCCCGAAAGTCAAACAGTGGCCGCTGACCGAAGTGAAAATTAAAGCGCTG
61         G  M  D  G  P  K  V  K  Q  W  P  L  T  E  V  K  I  K  A  L

241       ACCGCGATTTGCGAAGAAATGGAAAAAGAAGGCAAAATCACCAAAATCGGCCCGGAAAAC
81         T  A  I  C  E  E  M  E  K  E  G  K  I  T  K  I  G  P  E  N

301       CCGTATAACACCCCGATCTTTGCGATCAAAAAAGAAGATAGCACCAAATGGCGTAAACTG
101        P  Y  N  T  P  I  F  A  I  K  K  E  D  S  T  K  W  R  K  L

361       GTGGATTTTCGCGAACTGAACAAACGCACCCAGGATTTTTGGGAAGTCCAACTGGGCATT
121        V  D  F  R  E  L  N  K  R  T  Q  D  F  W  E  V  Q  L  G  I

421       CCGCATCCGGCGGGCCTGAAGAAAAAGAAAAGCGTGACCGTGCTGGATGTCGGCGATGCG
141        P  H  P  A  G  L  K  K  K  K  S  V  T  V  L  D  V  G  D  A
481       TATTTTAGCGTGCCGCTGGATGAAGGCTTTCGCAAATATACCGCGTTTACCATCCCGAGC
```

```
161        Y  F  S  V  P  L  D  E  G  F  R  K  Y  T  A  F  T  I  P  S

541        ATCAACAATGAAACCCCGGGCATTCGCTATCAGTATAACGTGCTGCCGCAGGGCTGGAAA
181         I  N  N  E  T  P  G  I  R  Y  Q  Y  N  V  L  P  Q  G  W  K

601        GGCAGCCCGGCGATTTTTCAGGCGAGCATGACCAAAATTCTGGAACCGTTTCGCGCGAAA
201         G  S  P  A  I  F  Q  A  S  M  T  K  I  L  E  P  F  R  A  K

661        AACCCGGAAATCGTGATCTATCAGTATATGGCGGCGCTGTATGTGGGCAGCGATCTGGAA
221         N  P  E  I  V  I  Y  Q  Y  M  A  A  L  Y  V  G  S  D  L  E

721        ATTGGCCAGCATCGCGCGAAAATTGAAGAACTGCGCGAACATCTGCTGAAATGGGGCTTT
241         I  G  Q  H  R  A  K  I  E  E  L  R  E  H  L  L  K  W  G  F

781        ACCACCCCGGATAAAAAACACCAGAAAGAACCGCCGTTTTTATGGATGGGCTATGAACTG
261         T  T  P  D  K  K  H  Q  K  E  P  P  F  L  W  M  G  Y  E  L

841        CATCCGGATAAATGGACCGTCCAGCCGATTCAACTGCCGGAAAAAGATAGCTGGACCGTC
281         H  P  D  K  W  T  V  Q  P  I  Q  L  P  E  K  D  S  W  T  V

901        AACGATATTCAGAAACTGGTGGGCAAACTGAATTGGACCAGCCAGATTTATCCGGGCATT
301         N  D  I  Q  K  L  V  G  K  L  N  W  T  S  Q  I  Y  P  G  I

961        AAAGTGCGTCAGTTATGCAAACTGCTGCGTGGCACCAAAGCGCTGACGGATATTGTCCCG
321         K  V  R  Q  L  C  K  L  L  R  G  T  K  A  L  T  D  I  V  P

1021       CTGACGGAAGAAGCGGAACTGGAACTGGCGGAAAACCGCGAAATTCTGAAAGAACCTGTG
341         L  T  E  E  A  E  L  E  L  A  E  N  R  E  I  L  K  E  P  V

1081       CACGGCGTCTATTATGATCCGAGCAAAGATCTGATTGCGGAAATCCAGAAACAGGGCGAT
361         H  G  V  Y  Y  D  P  S  K  D  L  I  A  E  I  Q  K  Q  G  D

1141       GACCAGTGGACCTATCAGATCTATCAGGAACCGTTTAAAAACCTGAAAACCGGCAAATAT
381         D  Q  W  T  Y  Q  I  Y  Q  E  P  F  K  N  L  K  T  G  K  Y

1201       GCGAAACGTCGCACCACCCATACCAACGATGTGAAACAACTGACCGAAGCGGTGCAGAAA
401         A  K  R  R  T  T  H  T  N  D  V  K  Q  L  T  E  A  V  Q  K

1261       ATCAGCCTGGAAAGCATTGTGACCTGGGGCAAAACCCCGAAATTTCGCCTGCCGATCCAG
421         I  S  L  E  S  I  V  T  W  G  K  T  P  K  F  R  L  P  I  Q

1321       AAAGAAACCTGGGAAATCTGGTGGACCGATTATTGGCAGGCGACCTGGATTCCGGAATGG
441         K  E  T  W  E  I  W  W  T  D  Y  W  Q  A  T  W  I  P  E  W

1381       GAATTTGTTAATACCCCGCCGCTGGTGAAACTGTGGTATCAACTGGAAAAAGAACCGATT
461         E  F  V  N  T  P  P  L  V  K  L  W  Y  Q  L  E  K  E  P  I

1441       GCGGGCGCGGAAACGTTCCATGCATAA
481         A  G  A  E  T  F  H  A  *
```


Figure 2.10: The DNA coding and protein sequences of Tat (A), Nef (B) and RT (C) proteins fused to *LacZα*, constructed using DNAMAN software version 4.0 (Lynnon Biosoft, Canada). The *LacZα* sequences are given in pink and the Tat, Nef and RT sequences are given in green. ATG and TAA sequences are given in black and were used as translation START and STOP codons respectively**.**

2.5.4 Expression of HIV-1 Tat, Nef and RT by *Salmonella enterica* serovar Typhimurium

The expression of HIV-1 Tat, Nef and RT proteins by the recombinant *Salmonella enterica* serovar Typhimurium was evaluated by SDS-PAGE. The expected band sizes of the *β*-galactosidase-Tat, *β*-galactosidase-Nef and *β*-galactosidase-RT on the SDS-PAGE gels were 19 kDa, 30 kDa and 59 kDa, respectively. The protein bands for Tat, Nef and RT were visible on the Coomassie-stained SDS-PAGE gels (Figures 2.11, lane 2; Figures 2.12A, lane 2; Figures 2.13A, lane 2). The foreign proteins seemed to be most highly expressed bacterial proteins. Western blotting was further used to confirm the expression of Tat, Nef and RT proteins (Figures 2.12B; Figures 2.13B). The anti-Nef antibody reacted strongly with the Nef protein band (Figures 2.12B, lane 2). The anti-RT antibody also reacted with the RT band (Figures 2.13B, lane 2). However, the Western blot also showed that there were other smaller protein bands that reacted with RT antibody (Figure 2.13B, lane 2). No reactivity of the anti-Nef or anti-RT antibodies to the recombinant *Salmonella* carrying the empty plasmid (pUC19) was observed (Figures 2.12B, lane 3; Figures 2.13B, lane 3).

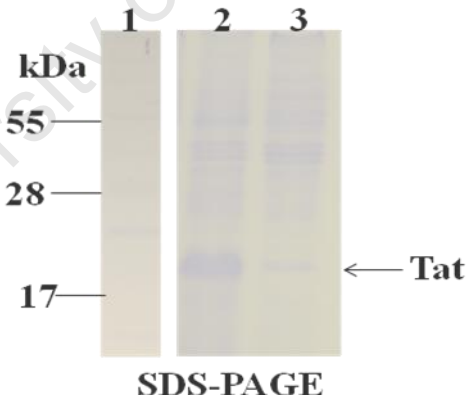

Figure 2.11: The SDS-PAGE gel showing the expression of the Tat protein by recombinant *Salmonella enterica* serovar Typhimurium. Lane 1: Marker, **Lane 2**: *Salmonella* expressing Tat, **Lane 3**: *Salmonella* carrying an empty plasmid.

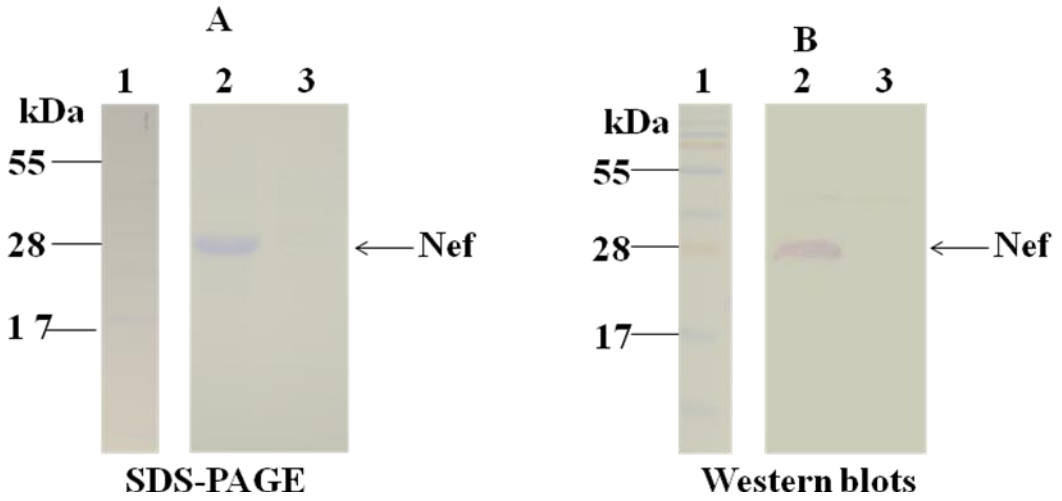

Figure 2.12: The expression of the Nef protein by recombinant *Salmonella enterica* serovar Typhimurium. A is a Coomassie-stained SDS-PAGE gel and **B** is a Western blot with Nef protein detected with anti-Nef antibody. **Lane 1**: Marker, **Lane 2**: *Salmonella* expressing Nef, **Lane 3**: *Salmonella* carrying an empty plasmid.

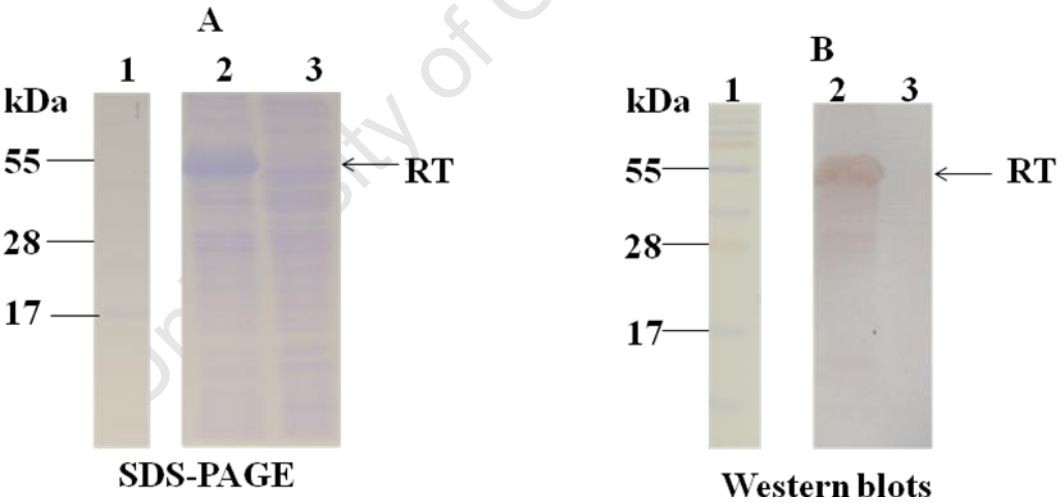

Figure 2.13: The expression of the RT protein by recombinant *Salmonella enterica* serovar Typhimurium. A is a Coomassie-stained SDS-PAGE gel and **B** is a Western blot with RT protein detected with anti-RT antibody. **Lane 1**: Marker, **Lane 2**: *Salmonella* expressing RT, **Lane 3**: *Salmonella* carrying an empty plasmid.

2.6 DISCUSSION

HIV-1 Tat, Nef and RT are important viral antigens which have previously been expressed in *E. coli* (Lader *et al*., 1987; Kaminchik *et al.,* 1990; Orsini *et al.,* 1996; Lebeko, 2007). In this study, HIV-1C *tat*, *nef* and *rt* genes derived from Southern Africa and targeted for AIDS vaccine development (Burgers *et al.,* 2006; Williamson *et al.,* 2003) were cloned and expressed in recombinant *Salmonella enterica* serovar Typhimurium mutant.

2.6.1 Cloning of HIV-1 subtype C *tat, nef* and *rt* genes

The *tat*, *nef* and *rt* genes were codon-optimized (by Geneart, Germany) for expression in *Salmonella enterica* serovar Typhimurium and were received in plasmids, pScript+Tat, pScript+Nef and pScript+RT respectively. The genes (*tat, nef* and *rt*) in these plasmids were cloned between the two restriction enzymes NarI and HpaI which generate sticky and blunt ends respectively. The initial objective of this study was to clone HIV-1 subtype C *tat, nef* and *rt* genes into a *Salmonella* prokaryotic expression system previously developed by Chin'ombe and colleagues (Chin'ombe *et al.,* 2009a). The expression system is based on the use of the *Escherichia coli* lactose (*lac*) operon domains in pGEM®-Teasy plasmid from Promega (USA) and antigen genes are fused in-frame with the 5' domain of the *β*-galactosidase *alpha* gene fragment and expression is under the *lac* promoter (Chin'ombe, 2007; Chin'ombe *et al*., 2009a, b). To achieve the first objective, the plasmid backbone (designated pGEM) for inserting the *tat, nef* and *rt* genes was generated. A recombinant plasmid, pGEM+SalmgagGFP, which contained the HIV-1 *gag* and *gfp* genes fused in-frame with the *β*-galactosidase *alpha* gene fragment, was initially digested with HindIII and the ends filled-up by the addition of *pfu* DNA polymerase and dNTPs. The addition of *pfu* DNA polymerase and dNTPs resulted in the blunting of the HindIII-generated sticky ends. The *gag* gene was removed from the plasmid backbone by digestion with NarI. The plasmid backbone at this stage now contained sticky and blunt ends and the *tat, nef* and *rt* genes were successfully cloned uni-directionally into the pGEM backbone after digestion from pScript+Tat, pScript+Nef and pScript+RT, respectively using NarI and HpaI. DNA sequencing of the recombinant plasmids, pGEM+Tat, pGEM+Nef, pGEM+RT was performed and

confirmed that the *tat*, *nef* and *rt* genes were successfully cloned in-frame with the 5' domain of the *β*-galactosidase *alpha* gene and were under the *lac* promoter.

2.6.2 Expression of Tat, Nef and RT proteins in *Salmonella enterica* serovar Typhimurium

An attenuated *Salmonella enterica* serovar Typhimurium was transformed using the recombinant plasmids (pGEM+Tat, pGEM+Nef, pGEM+RT) and the expression of Tat, Nef and RT proteins was evaluated. The Tat, Nef and RT proteins were the most highly expressed bacterial proteins by the recombinant *Salmonella enterica serovar* Typhimurium as their protein bands were visible on Coomassie-stained SDS-PAGE gels. The expression of Nef and RT by the recombinant *Salmonella enterica serovar* Typhimurium was further confirmed by Western blotting. The high-level expression of Tat, Nef and RT was in line with what was found in the previous studies by Chin'ombe and colleagues (Chin'ombe *et al*., 2009a). In these previous studies high levels of HIV-1 Gag, GFP and Gag-GFP were successfully expressed in attenuated *Salmonella enterica* serovar Typhimurium (Chin'ombe, 2007; Chin'ombe *et al*., 2009a, b). These previous reports together with findings in this study show that *Salmonella*, just like *E. coli*, may be used for recombinant antigen expression.

A number of factors, most of which were previously addressed by Chin'ombe, (2007) and colleagues, played a role in facilitating the high level expression of HIV-1 subtype C Tat, Nef and RT proteins in this study. These factors included gene codon optimization, fusion of the genes with the *LacZa* gene and the properties of the pGEM plasmid backbone. The codon optimization of the HIV-1 subtype C Tat, Nef and RT genes probably played a role in improving their expression by the *Salmonella* mutant. Several studies have shown that codon-optimization of heterologous genes improved their expression in bacterial systems (Apeler *et al*., 1997; Hu *et al.,* 2006; Hu *et al.,* 1989; Lakey *et al.,* 2000; Makoff *et al*., 1989; Yadava and Ockenhouse, 2003). The fusion of Tat, Nef or RT antigens to the *E coli β*-galactosidase *α*-fragment facilitated their high-level expression. In previous studies, HIV-Gag and GFP were expressed at higher levels than unfused Gag and GFP in recombinant *Salmonella* expression system (Chin'ombe, 2007). Other *E. coli* transcription and translation domains in the pGEM backbone such as transcription initiation, transcription termination, start codon (ATA), stop codon (TAA) and ribosomal

binding site were also recognized by the *Salmonella* bacteria and played some role in facilitating the expression of the Tat, Nef and RT proteins (Brown *et al.,* 1990; Poole *et al.,* 1995).

Recent studies by Chin'ombe *et al*. (2009a,b) showed that recombinant *Salmonella enterica serovar* Typhimurium expressing high levels of GFP and Gag antigens induced GFP specific CD8+ T cell responses and HIV-1 subtype C Gag specific cell mediated (CD4+ Th1 and Th2) responses in vaccinated mice. *Salmonella enterica serovar* Typhimurium expressing Gag antigen also induced HIV-1 subtype C Gag specific antibody responses in the sera of vaccinated mice (Chin'ombe *et al.*, 2009b). Therefore, the developed *Salmonella enterica* serovar Typhimurium expressing high levels of HIV-1 subtype C Tat, Nef and RT antigens in the current study may be used in future studies for the evaluation of immune responses in vaccinated mice.

The fact that the HIV-1 antigens in this study were expressed at very high levels, suggests that an attenuated recombinant *Salmonella* can potentially be used as a heterologous protein expression system for these proteins. Despite high levels of expression, the proteins could not be purified easily. Therefore, the decision was made to use His-tagged proteins for the development of Western blot to detect antibodies to HIV-1 subtype C infected individuals. This is described in Chapter 3 and 4.

In conclusion, HIV-1 subtype C *tat, nef* and *rt* genes were cloned and expressed at high levels in an attenuated strain of *Salmonella enterica* serovar Typhimurium. The recombinant *Salmonella* bacteria could be used as vehicles for delivery of the HIV-1 Tat, Nef and RT antigens. This needs to be investigated in future studies.

CHAPTER 3

PURIFICATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C TAT, NEF AND REVERSE TRANSCRIPTASE FROM *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

CONTENTS

3.1 INTRODUCTION

A number of expression systems are currently available for the production of recombinant proteins. They include bacterial, yeast, fungal, baculovirus and mammalian expression systems (Rai and Padh, 2001; Schmidt, 2004; Terpe, 2006). Bacterial expression systems, especially the *Escherichia coli* bacterium, are used extensively because of their advantageous properties (Hannig and Makrides, 1998; Jana and Deb, 2005; Sørensen and Mortensen. 2005). They can easily be cultured in a laboratory and grow rapidly to reach high biomass in a short period of time (Chen *et al.*, 2006; Langlais *et al.*, 2007; Terpe, 2006). They can be grown using cheaply available media (Terpe, 2006). The molecular biology of most bacterial systems is well-characterized and they can therefore easily be manipulated (Chen *et al.*, 2006; Langlais *et al.*, 2007; Terpe, 2006). There are many expression plasmid vectors available commercially which can be used for the cloning of foreign genes (Langlais *et al.*, 2007). There are some disadvantages of using bacteria as heterologous protein expression systems (Terpe, 2006). Proteins expressed in bacteria do not have post-translational modifications such as glycosylation. When over-expressed in bacteria, some foreign proteins form misfolded aggregations called inclusion bodies (Ventura and Villaverde, 2006). Foreign genes may also contain rare codons and may therefore need to be codon-optimized for optimal expression in bacteria (Das *et al*., 2009).

To facilitate the purification of recombinant proteins or antigens from bacterial expression systems, affinity tags such as Histidine tag (His-tag), arginine-tag (Arg-tag), glutathione S-transferase tag, calmodulin-binding tag, cellulose-binding domain and maltose-binding protein are normally used (Hochuli *et al*., 1988; Hopp *et al*., 1988; Sassenfeld and Brewer, 1984; Smith and Johnson, 1988; Stofko-Hann *et al*., 1992; Terpe, 2003). The His-tag is the most extensively used affinity tag for purification of foreign proteins expressed in bacterial systems (Terpe, 2003). His-tags are tandem repeats of 2-10 Histidine residues (usually 6) whose coding sequences are cloned in-frame to the gene to be expressed in the bacterial system. They efficiently bind to immobilized nickel nitrotriacetic acid (Ni-NTA) resins (Porath *et al.*, 1975; Siddappa *et al*., 2006). His-tagged foreign proteins can therefore be purified from bacteria by the metal-chelate affinity chromatography (Rank *et al.*,

2002). The His-tagged proteins bind to the immobilized Ni-NTA resins and can be eluted from the columns using specific buffers such as 20-250 mM imidazole (Hefti *et al*., 2001; Rank *et al*., 2002). His-tags can be fused at the C-terminus or N-terminus of the protein to be purified (Hochuli *et al*., 1988).

Although *E. coli* has been used extensively to produce foreign viral proteins, the closely-related bacteria such as *Salmonella* have not been investigated as potential heterologous expression systems. Since it has already been shown that HIV-1 Tat, Nef and RT proteins could be expressed at very high levels in recombinant *Salmonella enterica* serovar Typhimurium (Chapter 2), it was evident that the proteins could also be purified from the bacteria. The expressed HIV-1 Tat, Nef and RT proteins (Chapter 2) were required to be fused to an affinity purification tag such as His-tag to facilitate their purification from the recombinant *Salmonella enterica* serovar Typhimurium.

3.2 OBJECTIVES

The objective of this study was to purify the human immunodeficiency virus type 1 subtype C Tat, Nef and RT proteins previously expressed in recombinant *Salmonella enterica* serovar Typhimurium (Chapter 2). In order to purify the recombinant HIV-1 Tat, Nef and RT antigens from the bacteria, they (Tat, Nef and RT) were supposed to have the affinity purification His-tag. Recombinant expression plasmids which already contained *tat, nef* and *rt* genes fused to the His-tag coding sequences were therefore used. The plasmid constructs were pGEM+hisTat, pGEM+hisNef and pGEM+hisRT and contained *tat, nef* and *rt* genes cloned from pScript+Tat, pScript+Nef and pScript+RT respectively (Lebeko, 2007).

3.3 MATERIALS

The plasmids which contained the HIV-1 subtype C *tat*, *nef* and *rt* genes fused to the His-tag coding sequence were provided by Dr N Chin'ombe (University of Cape Town). They were pGEM+hisTat, pGEM+hisNef and pGEM+hisRT (Figure 3.1 A-C) and they contained *tat*, *nef* and *rt* genes fused to the His-tag coding sequences to facilitate purification. The pGEM+hisGFP plasmid which contained His-tagged *gfp* gene was also provided by Dr Chin'ombe. The plasmids were previously made by our research group (Lebeko, 2007). Special solutions and buffers used in this study are given in the Appendix A.

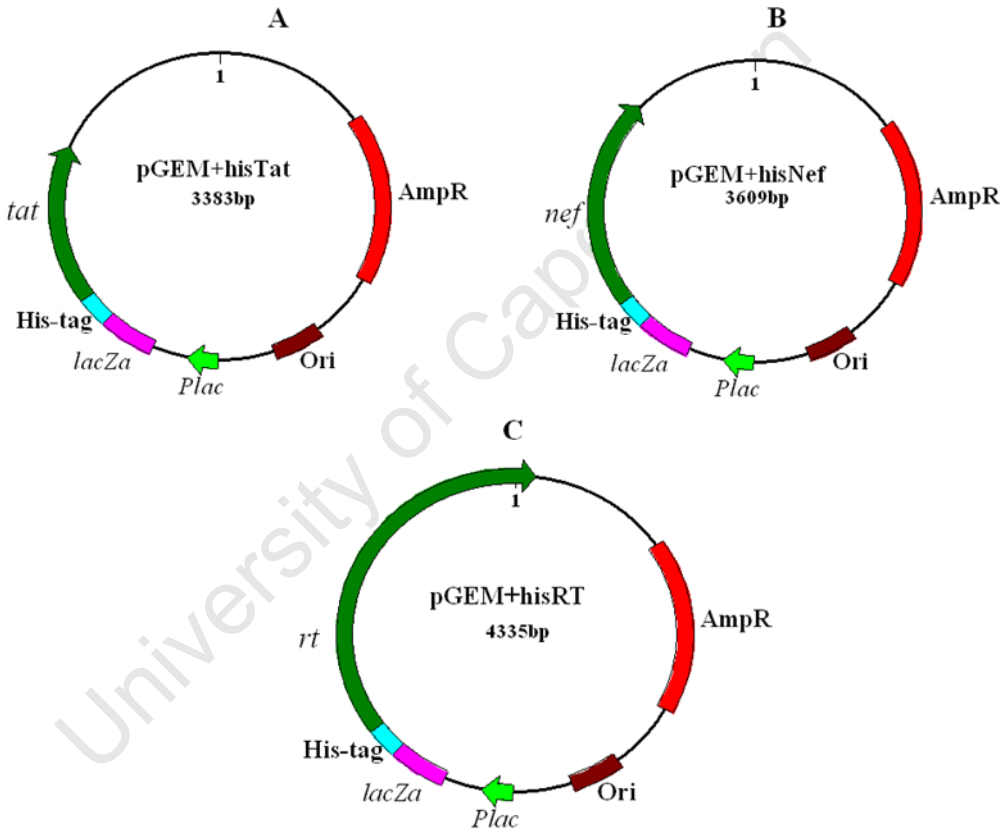

Figure 3.1: **Maps of HIV-1 subtype C *tat, nef* and *rt* expression plasmids (pGEM+hisTat, pGEM+hisNef and pGEM+hisRT),** constructed by DNAMAN software version 4.0 (Lynnon Biosoft, Canada) and were used in this study. The genes were previously cloned into the pGEM backbone and were fused in frame with the *β*-galactosidase α-gene (*lacZa*) and the His-tag. The expression of the fusion protein was the under upstream *E. coli lac* promoter**.**

3.4 METHODS

3.4.1 Restriction mapping of pGEM+hisTat, pGEM+hisNef and pGEM+hisRT

The pGEM+hisTat, pGEM+hisNef, and pGEM+hisRT plasmids which were provided were mapped to confirm the presence of the His-tagged HIV-1 *tat*, *nef* and *rt* genes. The plasmids were mapped using NotI and NdeI restriction enzymes. The digestion was done in a total volume of 20 µl and it contained 500 ng of plasmid DNA, 2X of 10X restriction buffer (Roche Diagnostics, Germany), 2 U of enzyme/s. The digestions were incubated at 37°C for 2 hrs and the resulting DNA fragments were analysed by 1.5% agarose electrophoresis.

3.4.2 Expression of His-tagged HIV-1 Tat, Nef and RT in recombinant *Salmonella enterica* serovar Typhimurium

Competent *Salmonella enterica* serovar Typhimurium cells were transformed with pGEM+hisTat, pGEM+hisNef, and pGEM+hisRT as previously described (Chapter 2). Total bacterial proteins containing His-tagged Tat, Nef and RT were extracted as previously described (Chapter 2, section 2.4.9). The protein samples were evaluated using the 12.5% SDS-PAGE as previously described (Chapter 2, section 2.4.11). A spectrum broad range colour stained marker (Bio-Rad, Canada) was loaded to identify the protein sizes. The expression of His-tagged Tat, Nef and RT was confirmed by Western blotting with mouse penta anti-His primary antibody (QIAGEN, Germany) at a 1: 500 dilution as previously described (Chapter 2, section 2.4.12 – 2.4.13). The expression of Nef and RT proteins was confirmed with mouse anti-Nef (Sigma, Germany) and rabbit anti-RT (Sigma, Germany) antibodies diluted 1: 1000. The primary mouse anti-His and anti-Nef antibodies were detected with secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Sigma, Germany) and rabbit anti-RT antibody was detected with goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Sigma, Germany). The total *Salmonella enterica* serovar Typhimurium extracts previously expressed Tat, Nef and RT proteins with no His-tag were used as controls. The total *Salmonella* extracts expressing an empty plasmid (pUC19) was used as negative control.

3.4.3 Purification of HIV-1 Tat, Nef and RT antigens under denaturing conditions

The His-tagged Tat, Nef and RT recombinant proteins were purified from recombinant *Salmonella enterica* serovar Typhimurium using Ni-NTA nickel chelate affinity chromatography. Briefly, the *Salmonella enterica* serovar Typhimurium were transformed with pGEM+hisTat, pGEM+hisNef, and pGEM+hisRT plasmids and grown as previously described (Chapter 2, section 2.4.8). Two volumes of bacterial culture (100 ml each) were pelleted at 5000 rpm (Sorvall RC-5C Plus superspeed centrifuge, Rotor S/N 10300651, USA) for 15 min at 4^{o}C. The pellets were washed with 50 ml cold PBS (pH 7.4) at 10000 rpm (Sorvall RC-5C Plus superspeed centrifuge, Rotor S/N 10300651, USA) for 30 min. One pellet was kept at 4^{o}C for purification of proteins under native conditions (Section 3.4.4) and another pellet was resuspended in 10 ml of denaturing lysis buffer (Appendix A: 38).

The cell suspension was incubated at room temperature for 60 min and swirled at 15 min intervals. The cell lysates were centrifuged at 12000 rpm (Sorvall RC-5C Plus superspeed centrifuge, Rotor S/N 10300651, USA) for 30 min at 24^{o}C to pellet the cellular debris and 10 ml supernatant was collected for affinity purification. The Fast start Column (QIAGEN, Germany) was prepared by incubation with 400 µl nickel-nitrilotriacetic acid (Ni-NTA) agarose buffer (QIAGEN, Germany) and 10 ml supernatant was applied to the column. The column was washed twice with 4 ml denaturing wash buffer (Appendix A: 39). The protein fraction was eluted seven times with 400 µl denaturing elution buffer (Appendix A: 40). The His-tagged GFP protein was also purified in parallel with Tat, Nef and RT proteins under denaturing conditions and served as a control. Samples were collected at each stage of the whole process for future SDS-PAGE analysis and Western blotting.

3.4.4 Purification of HIV-1 Tat, Nef and RT antigens under native condition

The Tat, Nef and RT proteins were also purified under native conditions using the same technique, Ni-NTA nickel chelate affinity chromatography. The His-tagged GFP protein was purified in parallel with Tat, Nef and RT proteins, respectively and served as a purification control. Briefly, the frozen pellets prepared from above (section 3.4.3) were thawed for 10 min at room temperature and resuspended in 10 ml of native lysis buffer (Appendix A: 41). The cells were lysed on ice for 3 hrs. The

cell lysate was centrifuged at 12000 rpm (Sorvall RC-5C Plus superspeed centrifuge, Rotor S/N 10300651, USA) for 30 min at 4^{o}C to pellet the cellular debris and 10 ml supernatant was collected for affinity purification. The Fast start Column (QIAGEN, Germany) was prepared by incubation with 400 µl NTA agarose buffer (QIAGEN, Germany) and 10 ml supernatant was applied to the column. The column was washed twice with 4 ml of native wash buffer (Appendix A: 42). The protein fraction was eluted seven times with 400 µl of native elution buffer (Appendix A: 43). At each stage, samples were collected for future SDS-PAGE and Western blotting.

3.4.5 Quantification of purified HIV-1 Tat, Nef and RT proteins

The fraction samples were quantified using the Nanodrop DC-1000 UV/VIS spectrophotometer (Thermo Scientific, USA) and BSA standard curves as described earlier (Chapter 2, section 2.5.14).

3.4.6 SDS-PAGE and Western blotting analysis of purified HIV-1 Tat, Nef and RT proteins

The purity of each of the HIV-1 Tat, Nef and RT proteins was evaluated using 12.5% SDS-PAGE and Western blotting. Twenty microlitres of each fraction was mixed with an equal amount of protein loading buffer and 15 µl of each fraction was loaded on 12.5% SDS-PAGE. A multicolour broad range protein marker (Fermentas, Canada) was loaded for identification of the protein bands. The proteins were electroblotted to a PVDF membrane (Amersham, UK) as previously described (Chapter 2, section 2.4.13). The blots were incubated in blocking buffer (Appendix A: 30) as previously described and purified HIV-1 Tat, Nef and RT proteins were detected with mouse penta anti-His primary antibody (QIAGEN, Germany) diluted 1: 500 as previously described (Chapter 2, section 2.4.14). The blots were incubated in goat anti-mouse IgG alkaline phosphatase-linked secondary antibody (Sigma, Germany) diluted 1: 10000 as previously described (Chapter 2, section 2.4.14). The blots were developed using NBT/BCIP (Roche Diagnostics, Germany) and scanned (CanonScan 8000F, China).

3.5 RESULTS

3.5.1 Verification of pGEM+hisTat, pGEM+hisNef and pGEM+hisRT

The presence of HIV-1 *tat, nef* and *rt* genes in pGEM+hisTat, pGEM+hisNef and pGEM+hisRT was confirmed by restriction mapping. The plasmids contained HIV-1 *tat, nef* and *rt* genes between the NotI and NdeI sites. The previously constructed pGEM+Tat, pGEM+Nef and pGEM+RT plasmids (Chapter 2) which contained the HIV-1 genes between EcoRI were used as controls. The expected sizes of the gene fragments after restriction digestion were observed (Table 3.1, Figures 3.2 A-C).

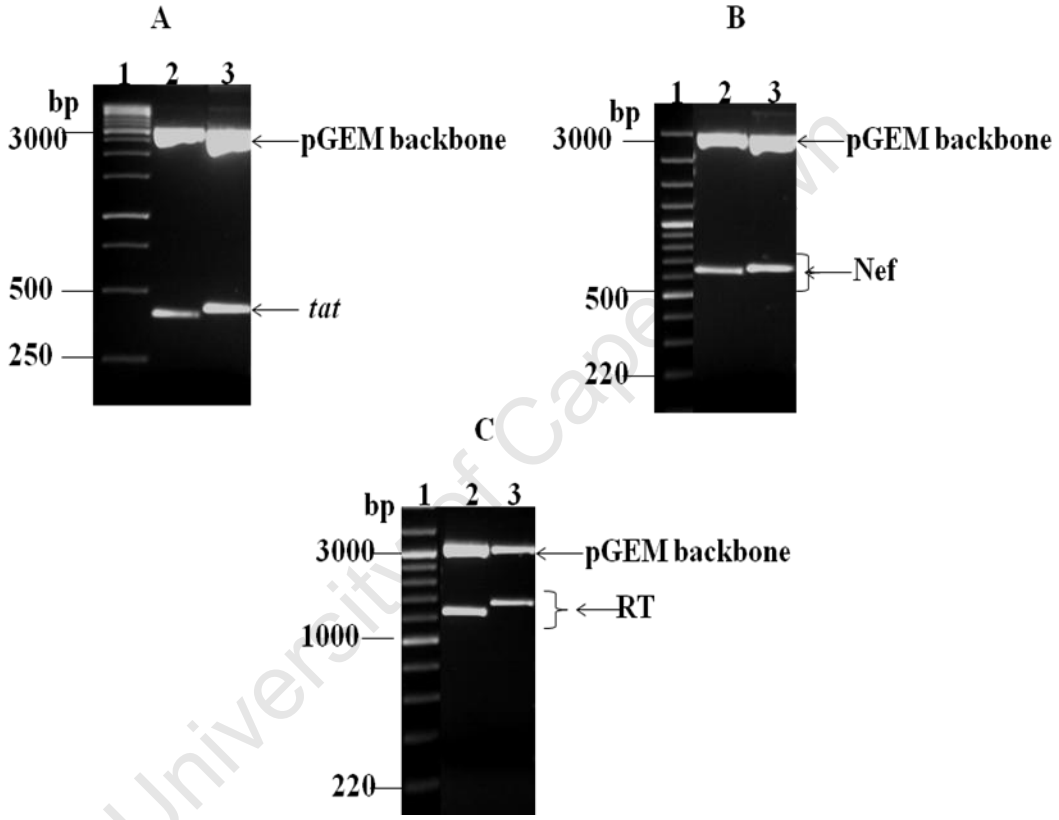

Figure 3.2: **Restriction mapping of pGEM+hisTat, pGEM+hisNef and pGEM+hisRT plasmids using NotI and NdeI.** (**A)** A pGEM+hisTat and pGEM+Tat**. Lane 1**: O'Generuler 100 bp DNA ladder, **Lane 2:** pGEM+hisTat, **Lane 3**: pGEM+Tat (control). **(B)** A pGEM+hisNef and pGEM+Nef**. Lane 1**: O'Generuler 100bp DNA ladder, **Lane 2:** pGEM+hisNef, **Lane 3**: pGEM+Nef (control). **(C)** A pGEM+hisRT and pGEM+RT**. Lane 1**: O'Generuler 100 bp DNA ladder, **Lane 2:** pGEM+hisRT, **Lane 3**: pGEM+RT (control).

Table 3.1: The expected bands of pGEM+hisTat, pGEM+hisNef and pGEM+hisRT after restriction mapping

Plasmid Name	Restriction Enzymes	Expected DNA band sizes (bp)
pGEM+hisTat	NotI and NdeI	403 and 2980
pGEM+Tat	EcoRI	429 and 2997
pGEM+hisNef	NotI and NdeI	629 and 2941
pGEM+Nef	EcoRI	655 and 2997
pGEM+hisRT	NotI and NdeI	1355 and 2980
pGEM+RT	EcoRI	1381 and 2997

3.5.2 Expression of His-tagged Tat, Nef and RT in *Salmonella enterica* serovar Typhimurium

Recombinant *Salmonella enterica* serovar Typhimurium was transformed with pGEM+hisTat, pGEM+hisNef and pGEM+hisRT plasmids for expression. Total *Salmonella* proteins containing His-tagged Tat, Nef and RT, respectively, were extracted. The expression of His-tagged Tat, Nef and RT proteins was analysed by SDS-PAGE and Western blotting. The total *Salmonella* protein extracts expressing non-tagged Tat, Nef and RT proteins, respectively, were used as controls. The protein bands for Tat, Nef and RT proteins were visible on the Coomassie-stained SDS-PAGE gels (Figure 3.2A, Figure 3.3A and Figure 3.4A).

The penta anti-His antibody recognizes an epitope comprising five consecutive Histidine residues. Western blot analysis with this antibody detected only His-tagged Tat, Nef and RT proteins (Figure 3.2B, lane 2; Figure 3.3B, lane 2 and Figure 3.4B, lane 2). As expected, non-tagged (Tat, Nef and RT) proteins and total *Salmonella* extracts carrying an empty plasmid were not seen on the Western blots (Figure 3.2B, lanes 3 & 4; Figure 3.3B, lanes 3 & 4 and Figure 3.4B, lanes 3 & 4). The anti-Nef and anti-RT antibodies detected Nef and RT respectively (Figure 3.3C, lanes 2 & 3 and Figure 3.4C, lanes 2 & 3). Extra banding pattern were also detected by anti-RT

antibody. His-tagged Tat, Nef and RT proteins were observed with expected molecular weights of 20 kDa, 29 kDa and 58 kDa, respectively.

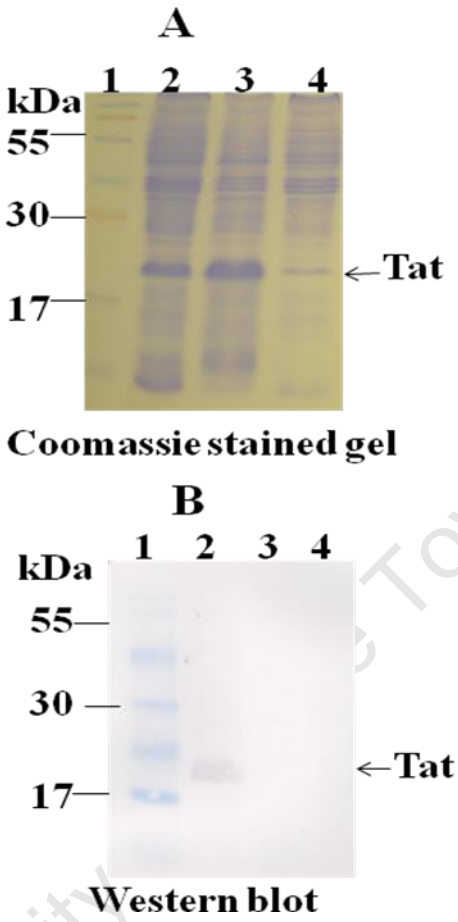

Figure 3.2: The expression of the His-tagged Tat protein by recombinant *Salmonella enterica* serovar Typhimurium. A is a Coomassie-stained SDS-PAGE gel and **B** is a Western blot with Tat protein detected with anti-His antibody. **Lane 1**: Marker, **Lane 2**: *Salmonella* expressing His-tagged Tat, **Lane 3**: *Salmonella* expressing non-tagged Tat (control), **Lane 4**: *Salmonella* carrying an empty plasmid (control).

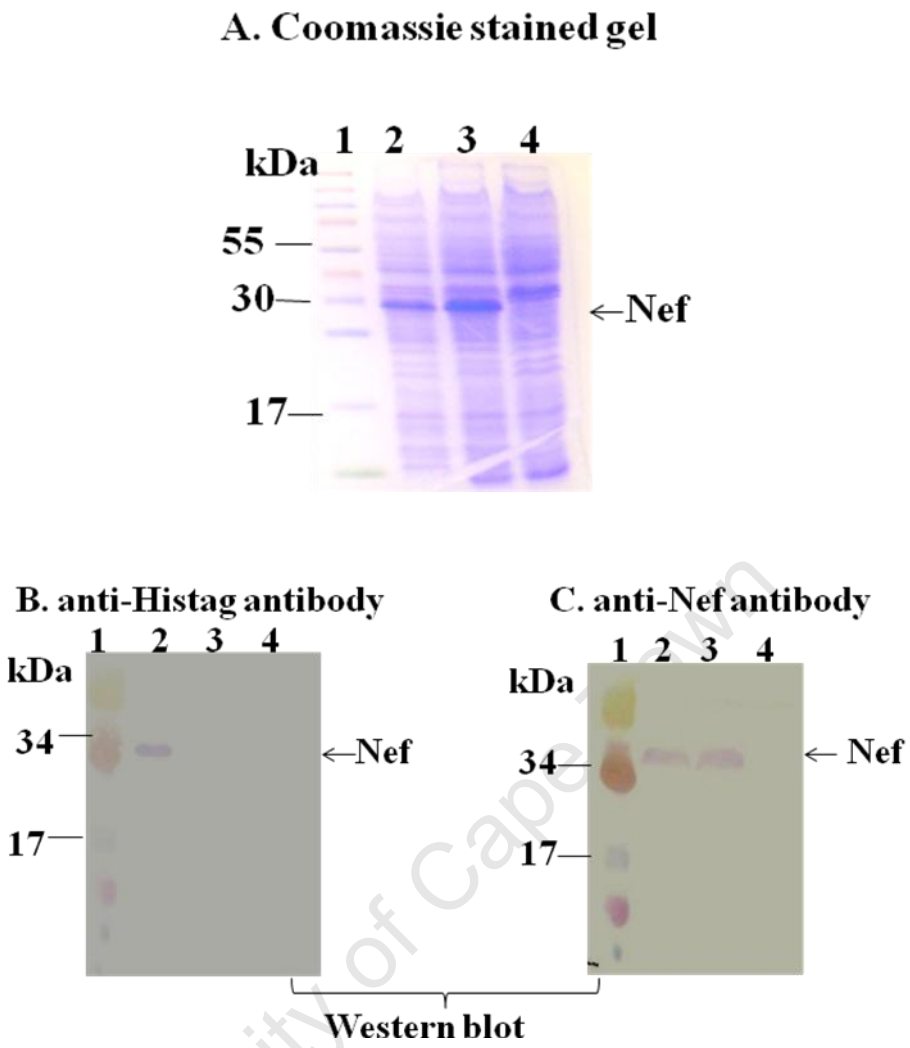

Figure 3.3: **The expression of the His-tagged Nef protein by recombinant *Salmonella enterica* serovar Typhimurium. A** is a Coomassie-stained SDS-PAGE gel, **B** is a Western blot with Nef protein detected with anti-His antibody and **C** is a Western blot with Nef proteins detected with anti-Nef antibody. **Lane 1**: Marker, **Lane 2**: *Salmonella* expressing His-tagged Nef, **Lane 3**: *Salmonella* expressing non-tagged Nef (control), **Lane 4**: *Salmonella* carrying an empty plasmid (control).

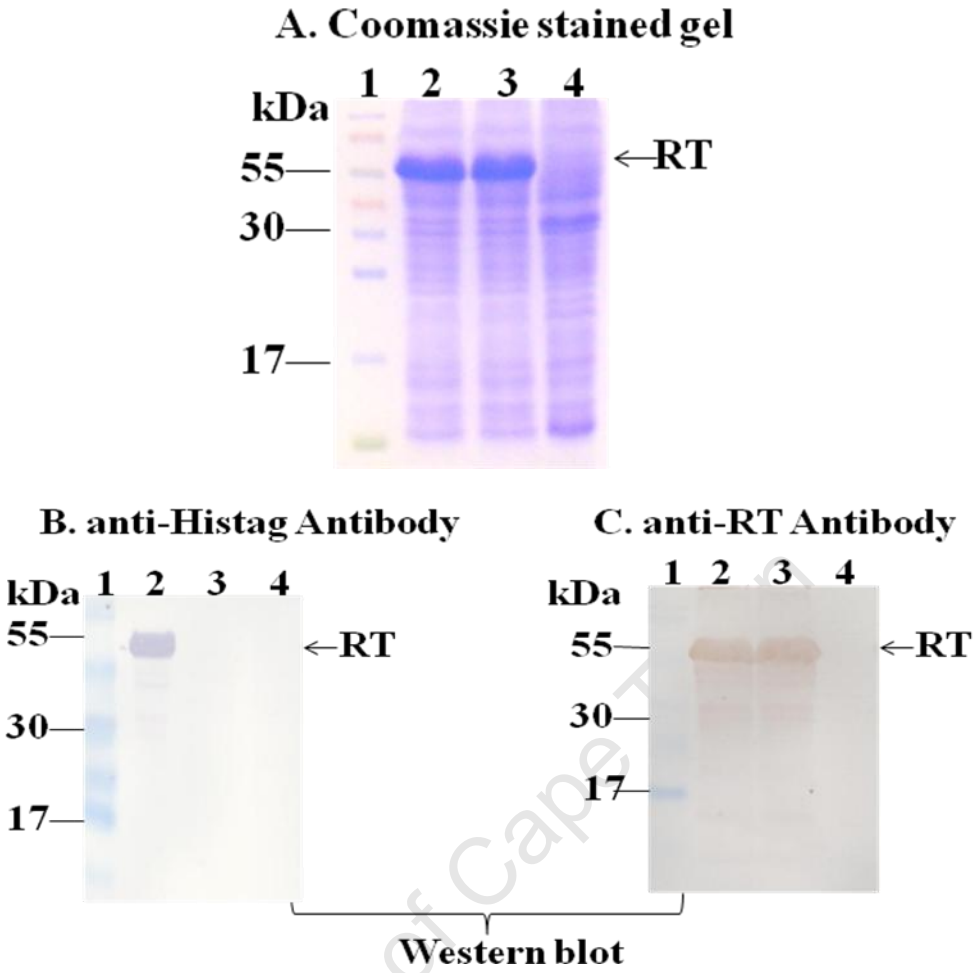

Figure 3.4: The expression of the His-tagged RT protein by recombinant *Salmonella enterica* serovar Typhimurium. A is a Coomassie-stained SDS-PAGE gel, **B** is a Western blot with RT protein detected with anti-His antibody and **C** is a Western blot with RT proteins detected with anti-RT antibody. **Lane 1**: Marker, **Lane 2**: *Salmonella* expressing His-tagged RT, **Lane 3**: *Salmonella* expressing non-tagged RT (control), **Lane 4**: *Salmonella* carrying an empty plasmid (control).

3.5.3 Purification His-tagged Tat, Nef and RT from *Salmonella enterica* serovar Typhimurium

The pGEM+hisTat, pGEM+hisNef and pGEM+hisRT plasmids were transformed into *Salmonella enterica* serovar Typhimurium for purification. His-tagged Tat, Nef and RT were purified under denaturing and native conditions using nickel chelate affinity chromatography with Ni-NTA agarose (QIAGEN, Germany). A 28 kDa GFP protein was also transformed in *Salmonella enterica* serovar Typhimurium and used as a positive purification control for Tat, Nef and RT, respectively. The concentration of the purified recombinant protein was determined by BSA standard curve. A 500 µg of GFP protein was obtained whereas 375 µg, 400 µg, and 300 µg of recombinant

Tat, Nef and RT proteins, respectively, were obtained by purification under denaturing conditions.

The purity and detection sensitivity of the purified recombinant Tat, Nef, RT and GFP (positive control) proteins were estimated following visualization on SDS-PAGE and Western blot. Coomassie-stained SDS-PAGE gels showed that recombinant GFP (positive control) was purified under both denaturing and native conditions (Figure 3.5 A-B, lanes 6-10). Nef protein was also purified under both denaturing and native conditions (Figure 3.6 A-B, lanes 6-10). Recombinant Tat and RT were purified under denaturing (Figure 3.7 A, lanes 6-8 and Figure 3.8 A, lanes 6-8) but not under native condition (Figure 3.7 B, lanes 6-8 and Figure 3.8 B, lanes 6-8). The purification of heterologous RT protein probably due to its high molecular weight was not as simple as of recombinant Tat, Nef and GFP proteins. The RT protein failed to be eluted with denaturing elution buffer adjusted to pH 4.5 as compared to Tat and Nef according to manufacturer's instructions (QIAGEN, Germany).

Western blot analyses with anti-His antibody showed that purified Nef and GFP (purification positive control) were detected in all elution fractions under denaturing and native conditions (Figure 3.5 C and D, lanes 6-10 and Figure 3.6 C and D, lanes 6-10) whereas purification of Tat and RT under denaturing conditions were detected only in elution fractions 1-3 (Figure 3.7C, lanes 6-8 and Figure 3.8C, lanes 6-8). Highest quantities of proteins were obtained in elution fraction 2 for Tat, Nef and GFP (Figure 3.5C, lane 7, Figure 3.6C, lane 7 and Figure 3.7C, lane 7) and elution fraction 1 for RT (Figure 3.7C, lane 6) purification under denaturing conditions. The purity of the proteins seemed to depend on the elution time points and protein quantities. There were extra bands observed under or below the bands representing Tat, Nef, RT and GFP proteins and these extra bands appeared mostly on the elution fractions containing the highest level of proteins purified under denaturing conditions (Figures 3.5C, 3.6C, 3.7C, lanes 7 and Figure 3.8C, lane 6). The expected molecular weights of Tat, Nef and RT proteins were 20 kDa, 29 kDa and 58 kDa, respectively and they seemed to be achieved except where the bands were skewed due to poor resolution of proteins on SDS-PAGE gel as seen in Figure 3.5D (lanes 6-10) and

Figure 3.6 B and D (lanes 6-10). The DNA and protein sequence of His-tagged Tat, Nef and RT proteins are given in Appendix B: 2.

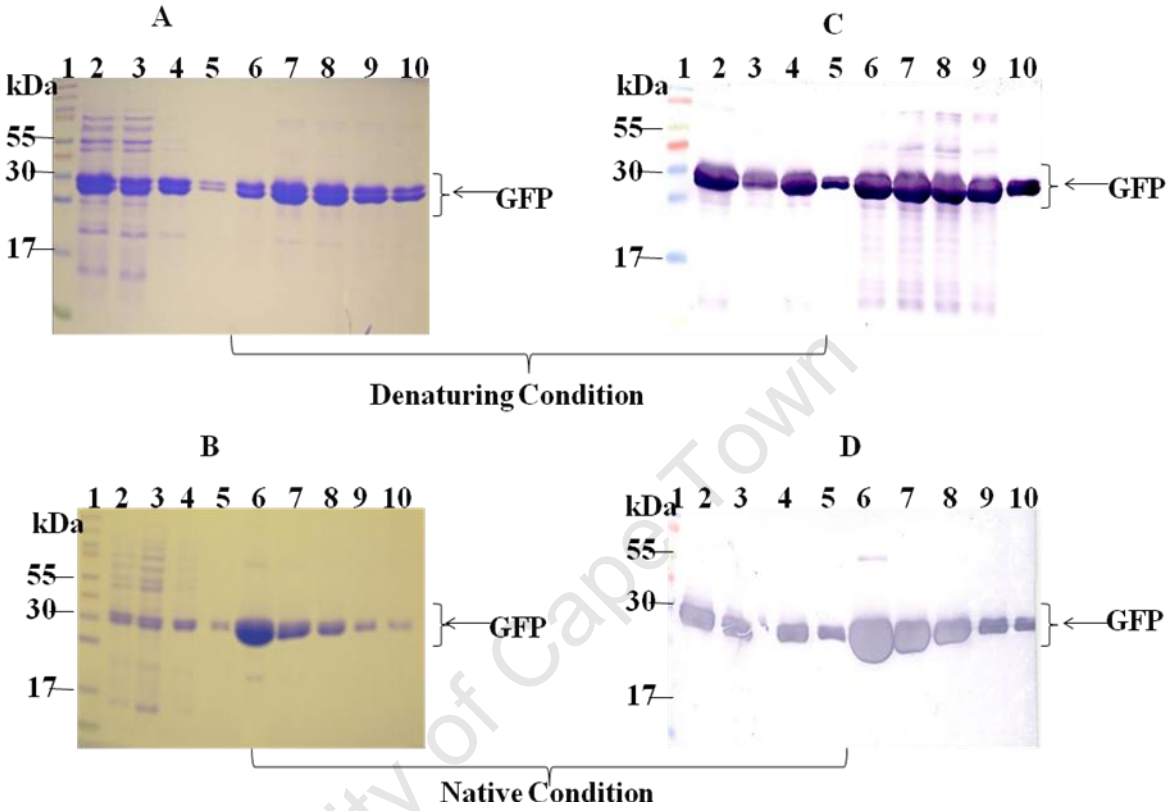

Figure 3.5: Purification of recombinant GFP from *Salmonella* enterica serovar Typhimurium. (A & B) Coomassie-stained SDS-PAGE showing GFP purified under (**A**) denaturing conditions and (**B**) native conditions. **(C & D)** Western blot showing GFP purified under **(C)** denaturing conditions and (**D)** native conditions. **Lane 1**: Protein marker**, Lane 2**: **CL** (Cell Lysates), **Lane 3: FL** (Flow Through) **Lane 4: W1** (Wash 1) **Lane 5:** W2 (Wash 2), **Lanes 6-10:** Elution Fractions 1-5.

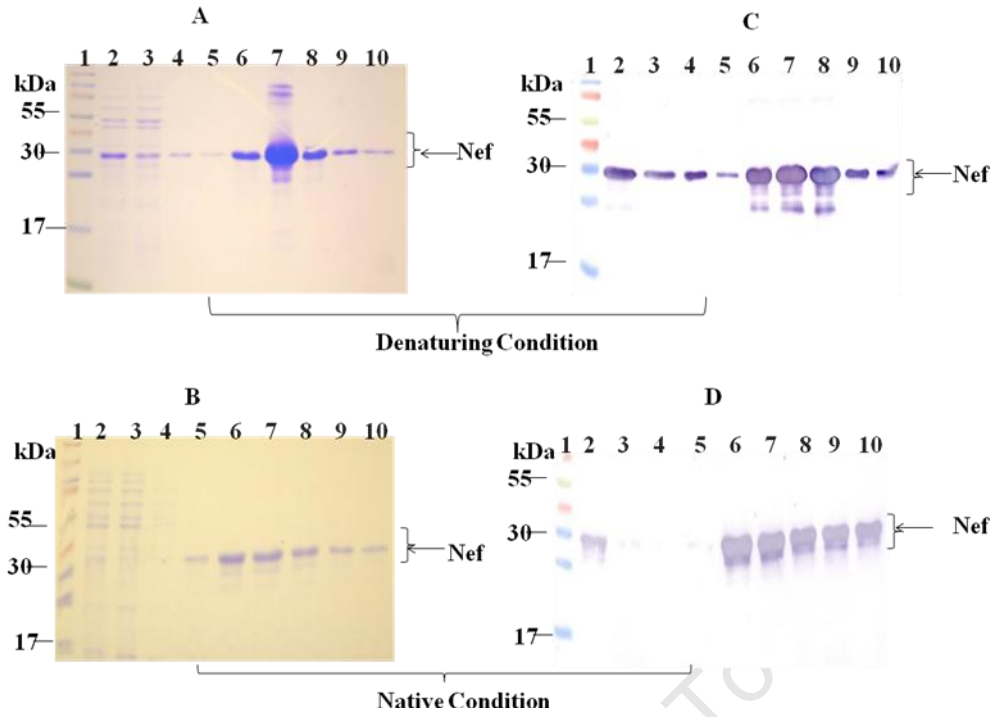

Figure 3.6: Purification of recombinant Nef from *Salmonella* enterica serovar Typhimurium. (A & B) Coomassie-stained SDS-PAGE showing Nef purified under (**A**) denaturing conditions and (**B**) native conditions. **(C & D)** Western blot showing Nef purified under **(C)** denaturing conditions and (**D)** native conditions. **Lane 1**: Protein marker**, Lane 2**: **CL** (Cell Lysates), **Lane 3: FL** (Flow Through) **Lane 4: W1** (Wash 1) **Lane 5:** W2 (Wash 2), **Lanes 6-10:** Elution Fractions 1-5.

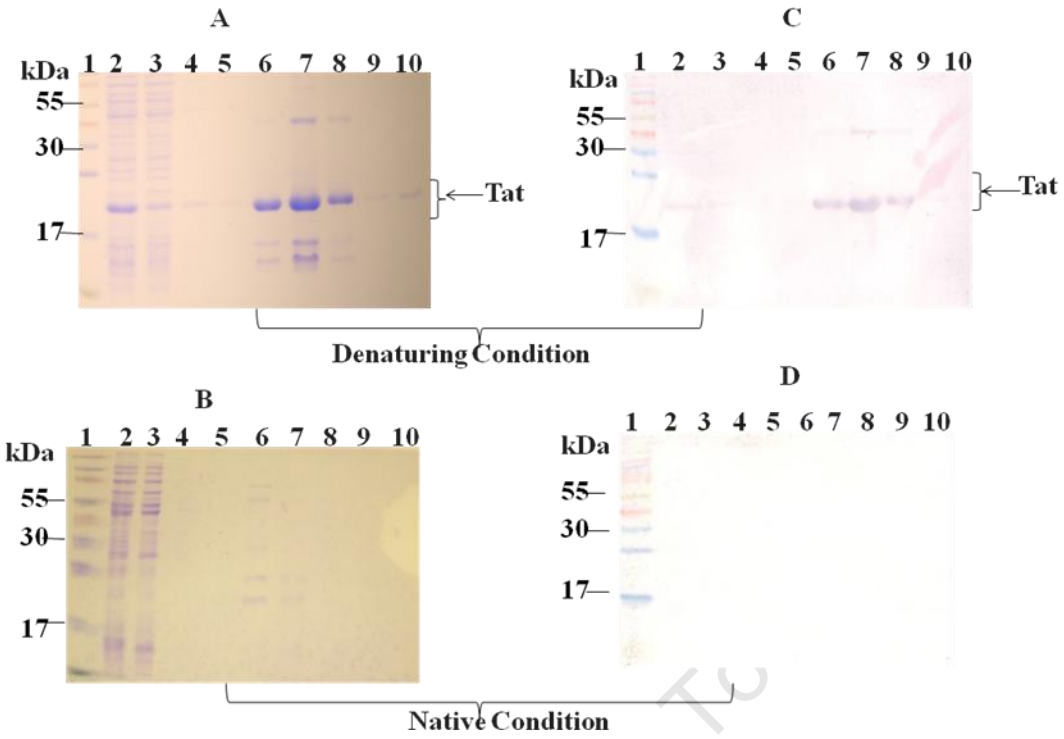

Figure 3.7: Purification of recombinant Tat from *Salmonella* enterica serovar Typhimurium. (A & B) Coomassie-stained SDS-PAGE showing Tat purified under (**A**) denaturing conditions and (**B**) native conditions. **(C & D)** Western blot showing Tat purified under **(C)** denaturing conditions and (**D)** native conditions. **Lane 1**: Protein marker**, Lane 2**: **CL** (Cell Lysates), **Lane 3: FL** (Flow Through) **Lane 4: W1** (Wash 1) **Lane 5:** W2 (Wash 2), **Lanes 6-10:** Elution Fractions 1-5.

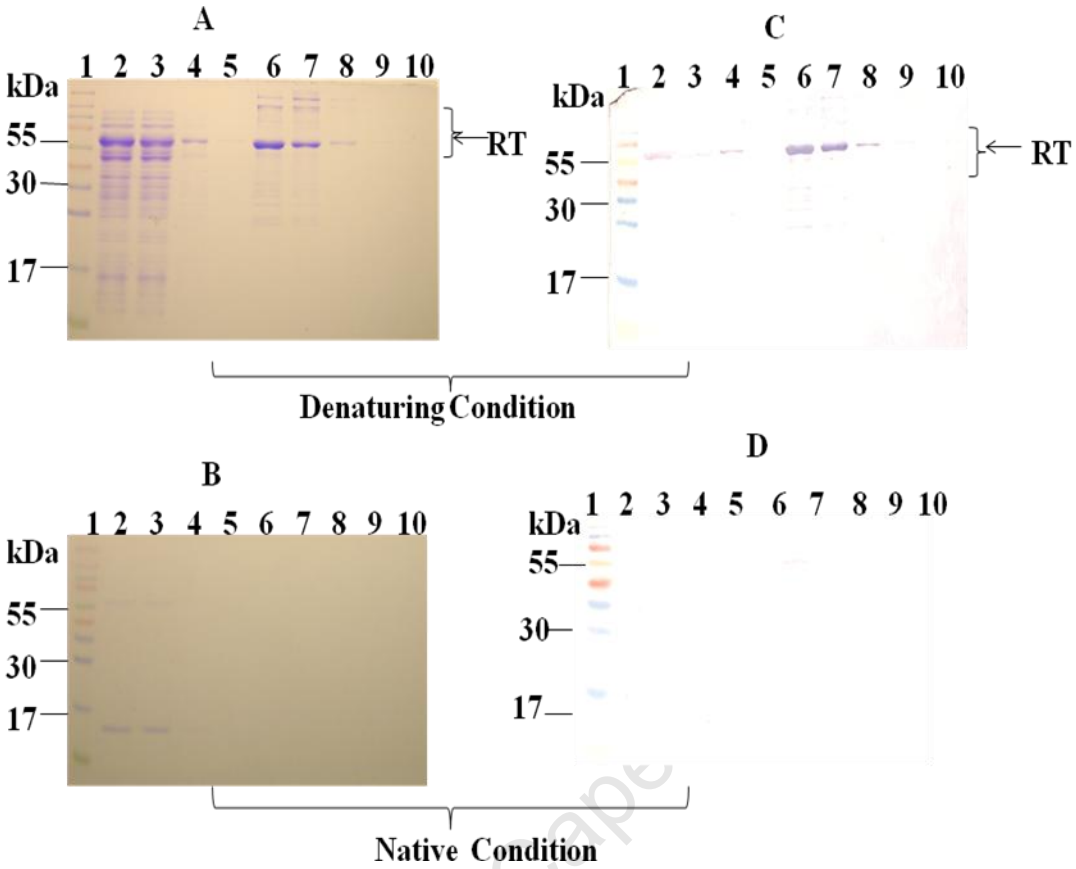

Figure 3.8: Purification of recombinant RT from *Salmonella* enterica serovar Typhimurium. (A & B) Coomassie-stained SDS-PAGE showing RT purified under (**A**) denaturing conditions and (**B**) native conditions.(C & D) Western blot showing RT purified under **(C)** denaturing conditions and (**D)** native conditions. **Lane 1**: Protein marker**, Lane 2**: **CL** (Cell Lysates), **Lane 3: FL** (Flow Through) **Lane 4: W1** (Wash 1) **Lane 5:** W2 (Wash 2), **Lanes 6-10:** Elution Fractions 1-5.

3.6 DISCUSSION

Recombinant HIV-1 Tat, Nef and RT proteins were previously expressed successfully at very high levels in recombinant *Salmonella enterica* serovar Typhimurium (Chapter 2). The expressed proteins could not be purified because they did not have purification tags such as the His-tag. In this study, the HIV-1 subtype C Tat, Nef and RT proteins which contained the His-tag were successfully purified from the recombinant *Salmonella enterica* serovar Typhimurium expressing them. The pGEM+hisTat, pGEM+hisNef and pGEM+hisRT were previously constructed (Lebeko, 2007) so that the coding sequences of the His-tag was fused in-frame between the *lacZα* and HIV-1 *tat, nef* or *rt* genes (Lebeko, 2007). The presence of the HIV-1 *tat, nef* or *rt* genes in the pGEM+hisTat, pGEM+hisNef and pGEM+hisRT respectively, was confirmed by restriction mapping using NotI and NdeI. This restriction mapping verified that the expected HIV-1 genes had previously been cloned into the given plasmids.

3.6.1 Expression of His-tagged Tat, Nef and RT in *Salmonella enterica* serovar Typhimurium

The pGEM+hisTat, pGEM+hisNef and pGEM+hisRT used to transform *Salmonella enterica* serovar Typhimurium and the expression of the HIV-1 Tat, Nef and RT was evaluated by SDS-PAGE and Western blotting. The protein bands of the three antigens were visible on the Coomassie blue-stained SDS-PAGE. This showed that the His-tagged HIV-1 antigens were most highly expressed *Salmonella enterica* serovar Typhimurium bacterial proteins. The intensities of the protein bands of the His-tagged HIV-1 antigens was almost the same as those of untagged HIV-1 ones. This suggested that the His-tag did not affect the expression of the HIV-1 antigens in recombinant *Salmonella*. Since the His-tag used in this study was small (only 6 Histidine residues), any negative impact on Tat, Nef or RT expression by the bacteria was anticipated. The expressed HIV-1 antigens were detected by the penta anti-His antibody in a Western blotting assay. This showed that the penta anti-His antibody (which recognizes any five consecutive Histidine residues) was able to bind to the hexa-Histag between the *LacZa* and the HIV-1 antigens.

3.6.2 Purification of His-tagged Tat, Nef and RT from *Salmonella enterica* serovar Typhimurium

The successful purification of proteins from recombinant bacteria depends mainly on whether their solubility, their localization and the accessibility of the His-tag. In most cases, proteins expressed in gram-negative bacteria such as *E. coli* or *Salmonella* form insoluble aggregations called inclusion bodies (Carrio *et al.*, 2001; LaVallie *et al.*, 1993; Makrides, 1996; Rattenholl *et al.*, 2001) and are difficult to purify. To successfully purify them, they have to be solubilised first using denaturants such as urea. In this study, the HIV-1 subtype C Tat, Nef and RT expressed from recombinant *Salmonella enterica* serovar Typhimurium were purified under both denaturing and native conditions using nickel chelate affinity chromatography. All the expressed antigens including GFP were successfully purified under denaturing conditions. However, only the GFP and the HIV-1 Nef were successfully purified under native conditions. This showed that a fraction of the expressed GFP and Nef was soluble and much of it was expressed as inclusion bodies and be solubilised under denaturing conditions with denaturant, urea. The failure to purify HIV-1 Tat and RT in this study suggested that they were predominantly expressed as inclusion bodies by the bacteria.

High levels of purified protein were obtained for Tat (375 µg) and Nef (450 µg) compared to RT (300 µg). Elution conditions for RT were changed from pH 4.5 to pH 3.7 and the protein was eluted in the first elution fraction whereas Tat and Nef were eluted in the second fraction. RT protein is a large protein of molecular weight of about 58 kDa and perhaps pH of 4.5 was too high to break down the bonds between the Ni-NTA and the His-tag to release the RT protein (QIAGEN purification kit booklet, Germany). The purity of the protein seemed to depend on the elution time points and quantity of recombinant Tat, Nef and RT proteins. There were non-specific bands observed in elution fractions during purification under denaturing condition and these were abundant in the highly concentrated elution fractions. This may represent contaminants associated with too much use of Ni-NTA matrix, His-tagged or truncated forms of His-tagged proteins, improper washing during purification, or proteolytic degradation products of the recombinant proteins in *Salmonella* bacteria. However, low concentration of imidazole (10 mM to 20 mM) in the lysis and washing buffer may aid in minimizing nonspecific binding and

reducing the amount of contaminating proteins. Reduction of the amount of Ni-NTA magnetic agarose beads, addition of protein inhibitors such as PMSF and keeping the protein buffers at 0-4ºC may also be necessary to prevent protein degradation (Schmitt *et al.*, 1993).

The results in this study were in line with previous findings in which HIV-1 Tat, Nef and RT proteins were purified from *E. coli* (Lebeko, 2007). It was shown that Tat and RT were purified only under denaturing conditions while Nef was purified under both denaturing and native conditions (Lebeko, 2007). In other studies, GFP and human proteins, Tat, Nef and RT labelled with His-tag were successfully purified from *E. coli* under denaturing conditions (Federico *et al*., 2001; LcGrice and Grüninger-Leitch, 1990; Ma *et al*., 2006a; Park *et al*., 2002; Woestenenk *et al.*, 2004). The GFP, Nef and Kinesin were also labelled with His-tag and purified under native conditions from *E. coli* (Blanc *et al.*, 1999; Finzi *et al*., 2003; Gibert *et al.*, 2000; Marusic *et al.*, 2007; Seleem *et al.*, 2008). The GFP was also successfully purified under denaturing conditions from *Salmonella enterica* serovar Typhimurium (Seleem *et al*., 2008).

In conclusion, high amounts of HIV-1 subtype C Tat, Nef and RT, which were mainly expressed as inclusion bodies, were purified from recombinant *Salmonella enterica* serovar Typhimurium under denaturing conditions. The purified proteins were useful as reagents in immunoassays for the detection of HIV-1 antibodies in clinical specimens (Chapter 4) or in animals vaccinated with HIV-1 vaccines (Chapter 5). The purified HIV-1 subtype C Tat, Nef and RT recombinant proteins can also be used to develop protein-based (subunit) vaccines for induction of antibody responses against HIV-1 infection in animal models.

CHAPTER 4

EVALUATION OF ANTIBODY RESPONSES IN HUMAN SERA

CONTENTS

4.2 INTRODUCTION

Humoral responses to HIV-1 infection lead to the production of antibodies against the virus. This process is aided by type 2 cytokines which are thought to enhance the humoral antibody responses in HIV infected individuals (Clerici and Shearer, 1996; Romagnani *et al.*, 1995; Swain *et al*., 1991). Neutralizing antibodies target the HIV envelope where they usually recognize the V3 loop of gp120, the CD4 binding sites and glycoprotein gp41 protein (Letvin and Walker, 2003; Parren and Burton, 2001). However, it should be noted that nearly 50% of the molecular weight of the envelope gp120 is covered with carbohydrate mannoses (glycan shield) that mask important epitopes from being recognised by neutralizing antibodies (Balzarin *et al*., 2005; Reitter *et al*., 1998). The CD4 binding sites and gp41 domains are highly conserved and antibodies to these sites recognise different HIV-1 subtypes (Letvin and Walker, 2003; Trkola *et al*., 1995).

Sera from HIV-infected individuals in South Africa and Uganda have been observed to neutralise different HIV-1 subtypes (Fanales-Belasio *et al*., 2002b). However, neutralizing antibodies may also bind weakly to CD4 binding sites and thus fail to neutralize the HIV infection (Wu et *al.*, 2009 Neutralizing antibodies have little or no effect on HIV-1 replication once the viral binding and fusion with the plasma membrane of the target cell has occurred (Clerici and Shearer, 1996; Letvin and Walker, 2003; Wu *et al*., 2009). Published studies by Carotenuto *et al.* (1998) and Pilgrim *et a1*. (1997) showed that broadly neutralizing antibodies protected long-term non-progressors against AIDS by delaying the disease progression as compared to rapid disease progressors. Another study reported that high neutralizing antibody titers in mothers may reduces the risks of mother-to-child HIV-1 transmission (Colgnesi *et al*., 1997; Kliks *et al*., 1994).

HIV infection induces the production of humoral antibodies in response to HIV proteins including structural and regulatory proteins (Letvin *et al.*, 1985). Humoral (both binding and neutralizing) antibodies may or may not lead to killing the virus (Clerici and Shearer, 1996). Neutralizing antibodies play an important role in establishing the viral containment in HIV exposed individuals, especially if they are present in high titers (Letvin *et al.*, 1985). This suggests that neutralizing antibody

responses would be a good target for the development of prophylactic vaccine. This is supported by two findings; (1) antibody responses have been elicited and correlated with the effectiveness of commercially licensed vaccines such as those for measles, mumps and rubella (Marin *et al.,* 2007; Vesikari *et al.,* 2004) and (2) specific anti-SHIV antibodies associated with protection against SHIV infection were observed in non-human primates (Belliard *et al.,* 2005).

The ELISA, RIA and Western blot assays are useful for evaluating the natural binding antibody responses to HIV (Bahraoui *et al.,* 1990; Chen *et al*., 1999; Demirhan *et al*., 1999). Indicated by a number of clinical studies, these techniques detected HIV-1 antibodies against viral proteins such as structural (Gag, Pol and Env), regulatory (Tat and Rev) and accessory (Nef, Vif, Vpr and Vpu) proteins in a cohort of HIV-1 infected individuals (Chen *et al.,* 1999; Chou *et al*., 1990; Lange *et al.,* 1986; Matsuda *et al*., 1988; Reiss *et al*., 1990). Although antibodies to regulatory proteins are less quantitavely detected as compared to structural proteins, there is evidence that they may elicit the antibody responses (Allan *et al*., 1985; Chen *et al.,* 1999; Lange *et al*., 1986; Keet *et al.,* 1994; Kühn *et al*., 1987). In an early study, Reiss *et al*. (1990) reported that low antibody response against regulatory proteins is associated with rapid disease progression after HIV-1 infection. It should be noted that this may be a marker of a lack of HIV control and not the actual cause of disease progression.

Binding antibody responses to core proteins such as Gag p24 and Gag p17 are detected in high prevalence in the sera of HIV-infected individuals (Chen *et al.,* 1999; Lange *et al.*, 1986; Keet *et al*., 1994). In contrast, antibodies to Tat, Nef and RT have been detected in lower prevalence and less frequently in the sera of individuals. However, antibodies to Tat and Nef antigens were associated with slow progression to AIDS (Chen *et al*., 1999; Reiss *et al,* 1990), suggesting the possibility of using the detection of these antibodies as a marker of HIV disease progression. Published studies showed that AIDS occurred mostly in individuals who were negative for anti-Nef and anti-Tat antibodies as compared to individuals who were positive to these antibodies (Chen *et al.,* 1999). However, these studies have been conducted in individuals infected with non-subtype C viruses. Similar studies using sera from individuals infected with HIV-1 may be useful in elucidating if such

associations occur among HIV-1 infected individuals in South Africa, where subtype C is the most prevalent subtype.

4.2 OBJECTIVES

Currently, no studies have been conducted on Tat, Nef or RT antibody responses in the sera of South African HIV-1 subtype C infected individuals. The HIV-1 Tat, Nef and RT play an important role in the viral life cycle and pathogenesis of the disease (Chang *et al.,* 1994; Havlir *et al.,* 1996; Wei *et al.,* 2003). Acquiring increased knowledge on the detection of antibody responses to Tat, Nef and RT antigens in the sera of HIV-1 subtype C infected individuals could help to determine whether the presence of antibodies to these antigens can serve as marker of disease progression. In addition, the efficient presence of antibody responses to Tat, Nef and RT antigens would also determine whether these antigens could be included in diagnostic tools for HIV infection. Therefore, this study was aimed at investigating the prevalence of antibody responses to purified HIV-1 Tat, Nef and RT in the sera of HIV-1 subtype C infected individuals (Chapter 3) by employing Western blot assay. In addition, the study investigated whether the prevalence of these antibodies is associated with the clinical stage of the AIDS disease as defined by the level of CD4 cells count in the peripheral whole blood and plasma HIV-1 viral load.

4.6.1 MATERIALS

Serum samples were obtained from a clinical study which investigated the prevalence of genital HPV infection among HIV-1 infected and uninfected individuals (Mbulawa *et al.,* 2009). The investigation was approved by the research ethics committee of the University of Cape Town. These individuals were tested at Gugulethu clinic with Rapid HIV antibody test (Manyanani Clinic, Empilisweni

centre, Cape town, SA) and gave their informed consent. Four- hundred and eighty one (481) serum samples were from HIV-1 infected males and females individuals aged between 17 and 65. Of these 481 samples, 124 pairs were from sexual partners. Some of the individuals had received antiretroviral therapy but the treatment record was unavailable as the records had not yet been transcribed into the central study database. All HIV positive individual in the study received a referral to the nearest ARV clinic to determine if they needed treatment. An additional 20 serum samples from HIV-1 negative individuals, men and women served as negative controls. The samples were categorised into groups according to donor's CD4+ T cell count in peripheral blood and plasma HIV RNA load as given in Table 4.1. Group A (CD4: 500 cells/µl or more) indicates healthy immune system, in spite of HIV-1 infection; Group B (CD4: 201 to 499 cells/µl) indicates damage of immune system due to HIV-1 infection and Group C (CD4: 200 cells/µl or less) indicates the weakening of immune system and possible onset of AIDS (Mellors *et al.*, 1997; Mylonakis *et al.*, 2001; Phair *et al*., 1990). All sera were heated at 56°C for 60 min to inactivate HIV (Spear *et al.*, 1991).

Table 4.1: Categories of sera samples into groups based on CD4+ T cell counts and plasma viral load

CD4 category (cells no/µl)	**No of samples**
Group A: CD4 500 or more	115
Group B: CD4 201 to 499	286
Group C: CD4 200 or less	80
Viral load category (RNA copies/ml)	**No of samples**
Group 1: VL 50 to 500	20
Group 2: VL 501 to 5000	56
Group 3: VL 5001 to 20, 000	77
Group 4: VL 20, 001 or more	129

4.6.1 METHODS

4.6.2 Establishment of the best equivalent amounts of proteins

In order to establish the optimum amount of each protein to load on the SDS-PAGE, molar equivalents of recombinant Tat, Nef and RT proteins previously purified (Chapter 3) were determined using the Promega *BioMath* program (http://www.promega.com/BioMath/calc05.htm). This was done in an attempt to

achieve the best and equivalent banding pattern of all the proteins to eliminate the background reactivity that could arise during screening of the sera. Briefly, the purified proteins were serially diluted at four 2-fold concentrations and subjected to 12.5% SDS-PAGE as previously described (Chapter 2, section 2.4.11). The proteins were transferred to the PVDF membrane (Amersham, UK) using electrophoretic blotting as previously described (Chapter 2, section 2.4.13). Recombinant Tat, Nef and RT proteins were detected with penta His-tag antibody as previously described (Chapter 2, section 2.4.14). After electroblotting, the SDS-PAGE gel was stained with Coomassie blue staining (Appendix A: 34) to allow the estimation of the transfer efficiency.

4.6.2 Establishment of the best dilution of anti-human IgG alkaline phosphatase conjugate

The best dilution of anti-human IgG alkaline phosphatase to detect the human sera was tested. Based on previous equimolar protein dilutions, two protein cocktails were prepared as follows: Cocktail 1 contained 0.022 µg Tat/well, 0.035 µg Nef/well & 0.06 µg RT/well and the second quantity of a cocktail contained 0.011 µg Tat/well, 0.18 µg Nef/well & 0.03 µg RT/well. The cocktails were migrated next to each other on 12.5% SDS-PAGE as previously described (Chapter 2, section 2.4.11). The proteins were electroblotted onto the PVDF membrane as previously described (Chapter 2, section 2.4.13) and the membrane was cut into strips. HIV positive control serum from New *Lav Blot* 1 commercial kit (Bio-Rad, Germany) was incubated with *Salmonella* (common gut pathogens) cell lysates overnight at 4^0C to remove antibodies to *Salmonella*. The strips were incubated with HIV positive control serum at a dilution of 1:100 for 2 hrs with gentle shaking at room temperature. The strips were washed four times (10 min each) with washing buffer (Appendix 30) to remove unbound antibodies and incubated with polyclonal anti-human IgG conjugated to alkaline phosphatase (Sigma, Germany) in various dilutions (1: 2000; 1: 4000; 1: 6000; 1: 8000 and 1: 10,000) for 60 min. The strips were washed as previously and developed with NBT-BCIP substrate solution for 15 min. Reaction was then stopped by rinsing the strips with water. The strips were dried with paper towel and scanned (CannonScan 8000F, China). The intensity of the reactive bands was compared and the optimal dilution was selected for use in further

experiments. The optimal dilution was selected based on the optimal band intensity for all antigens.

4.6.2 Detection of HIV-1 Tat, Nef and RT antibodies in human sera by Western blot assay

To detect the presence of HIV-1 Tat, Nef and RT antibodies in the sera of HIV-1 infected individuals, a cocktail containing purified recombinant proteins (Tat, Nef and RT) was migrated on a 12.5% SDS-PAGE as previously described (Chapter 2: section 2.4.11). The molar equivalent amounts of each protein used for SDS-PAGE was as follows: Tat (0.011 µg), Nef (0.018 µg) and RT (0.03 µg) as established in section 4.4.2. The proteins were transferred on to PVDF membranes as previously described (Chapter 2, section 2.4.13). Following transfer, the PVDF membranes were incubated with 5% blocking buffer (Appendix A: 30) for 2 hrs at room temperature and left at -4^{0}C on a shaker overnight. The membranes were dried and kept at 4^{0}C until use. Before use in a Western blot assays, the sera were incubated with *Salmonella* cell lysates at dilution of 1:100 overnight at 4^{0}C to remove the antibodies to *Salmonella*. The membranes were taken out of the fridge, cut into thin strips, labelled and soaked in methanol (for 2 min) and water for 5 min. After soaking, the strips were washed once for 10 min with washing buffer (Appendix A: 30) and each strip was incubated with serum at a dilution of 1:100 at room temperature for 2 hrs. Samples from HIV negative donors were used as negative controls. The strips were washed four times (10 min each) with washing buffer (Appendix 30) to remove unbound antibodies. The strips were then incubated with goat anti-human IgG conjugated to alkaline phosphatase (Sigma, Germany) at a dilution of 1:8000 (as established from the above section 4.4.2) for 60 min at room temperature. After incubation, the membranes were washed as before and the binding of the secondary antibody was detected with NBT-BCIP substrate solution (Roche Diagnostics, Germany) following the manufacturer's instructions. The reaction was allowed until 15 min and the strips were then rinsed with water, dried with paper towel, taped on white A4 paper and scanned (CanonScan 8000F, China).

Each Western blot strip was scored by visual examination as negative or positive for antibodies to Tat, Nef and RT antigens based on the absence or presence of a band

corresponding to the expected position of these antigens irrespective of the intensity of the reactive bands.

The overall performance of the Western blot assay method in detecting antibodies to HIV was estimated by calculating the sensitivity and specificity using the following formulas:

$$\textbf{Sensitivity} = \frac{\textbf{Number of positive samples to any of the 3 HIV proteins}}{\textbf{Number of total samples from HIV positive donors}} \times 100\%$$

$$\textbf{Specificity} = \frac{\textbf{Number of negative samples to any of the 3 HIV proteins}}{\textbf{Number of total samples from HIV negative donors}} \times 100\%$$

4.6.2 Statistical analysis

Statistical analysis was performed using Computer software, Graph Pad Prism version 5.0 (La Jolla, California, USA). The data were tabulated by 2 x 2 contingency tables to determine the *p-values*. A two-tailed Fisher's exact test (X^2 – test) determined whether there was significant difference between the two or among three groups. Significance was measured to a 95% confidence interval with *p-value* of $p \leq 0.05$ considered significant.

4.6.1 RESULTS

4.6.2 Establishment of the best equivalent amounts of proteins to give reproducible Western Blots

As previously described in section 4.2.1, the best molar equivalent amount of Tat, Nef and RT proteins was established so that human sera could be tested. Tat, Nef and

RT proteins were clearly visualized by Coomassie blue staining on the SDS-PAGE (Figure 4.1, A-C). After electroblotting, the SDS-PAGE gel was also stained with Coomassie blue to estimate the transfer efficiency and the results showed that Nef and RT were almost completely transferred while some Tat protein remained on the stained SDS-PAGE gel after transfer (Figure 4.1, D - E). Western blots showed that Tat, Nef and RT proteins were strongly detected with penta anti-His-tag antibody at all dilutions (Figure 4.1, G - I). When further serial dilutions of Tat, Nef and RT were prepared in a cocktail format, clear banding patterns were observed for cocktail number 1 that contained 0.022 µg Tat, 0.035 µg Nef & 0.06 µg RT and cocktail number 2 that contained 0.011 µg Tat, 0.018 µg Nef and 0.03 µg RT (Figure 4.2, Lane 2 & 3). Beyond these concentrations, bands corresponding to Tat, Nef and eventually to RT could not be visualized. Thus, the molar equivalent corresponding to 0.011 µg Tat, 0.018 µg Nef and 0.03 µg RT was chosen as the optimum amounts of proteins for cocktails in further Western blot assays. This combination gave clearly visible band on Western Blot with a relatively low background.

4.6.2 Establishment of the best dilution of anti-human IgG alkaline phosphatase conjugate

Five strips bound with two cocktails (cocktail 1 contained 0.022 µg Tat, 0.035 µg Nef & 0.06 µg RT and cocktail 2 contained 0.011 µg Tat, 0.018 µg Nef and 0.03 µg RT) were prepared and used for establishment of optimal dilution of anti-human IgG alkaline phosphatase to detect IgG antibodies in human sera. The Western blot assay showed that 1: 8000 dilution on the lane loaded a cocktail containing 0.022 µg, 0.035 µg and 0.06 µg of Tat Nef and RT, respectively, was the optimum concentration because it resulted in good detection levels with low background (Figure 4.3C, lane 8). Detection with positive HIV serum control *(LAV Blot* 1, Bio-Rad, USA) resulted in development of bands of varying intensity with RT giving the highest intensity and Tat being the lowest intensity, indicating weak responses to Tat antigen. In addition, there was low level of background on the blot which was eliminated by increasing the incubation time with S*almonella* lysates from 2 hrs to overnight at 4°C (data not shown). Subsequently, all incubation of sera with *Salmonella* lysates was done overnight at 4°C.

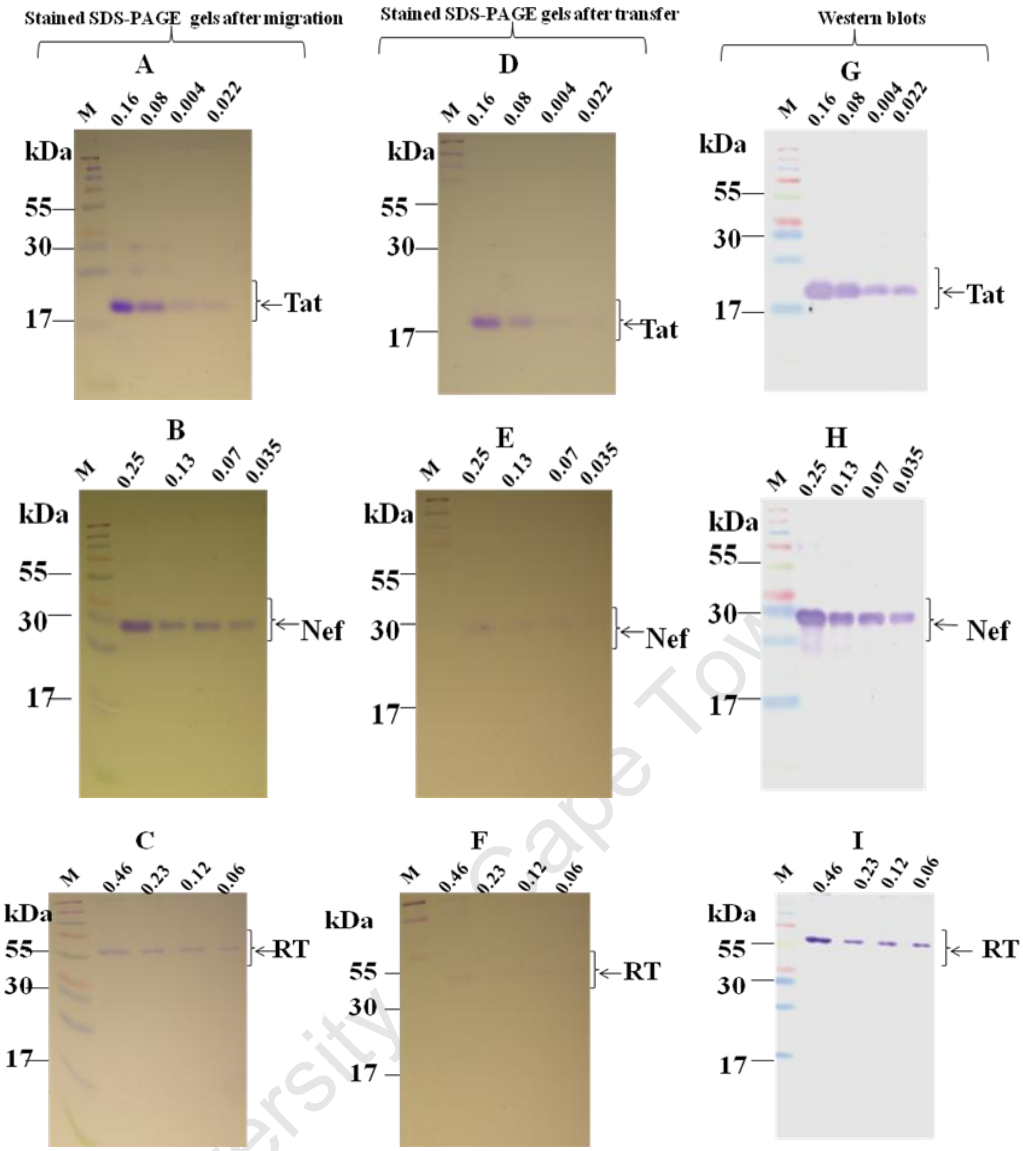

Figure 4:1 Coomassie stained SDS-PAGE and Western blot assay for the establishment of the optimum amount of proteins for cocktail preparation. Purified Tat, Nef and RT proteins were prepared in four 2-fold serial dilutions showed on top of each SDS-PAGE or blot and loaded onto 12.5% electrophoresis gel. The proteins were bound to the membrane and detected with anti-Histag antibody. Figures shows Coomassie-stained SDS-PAGE gels of Tat, Nef and RT proteins after migration (A-C), Coomassie-stained SDS-PAGE gels after transfer of the proteins to the membrane (D-F) and Western blot assays with anti-Histag antibody detection (G-I). Numbers on the top of the SDS-PAGE gels or Western blots represent dilution concentrations of the samples.

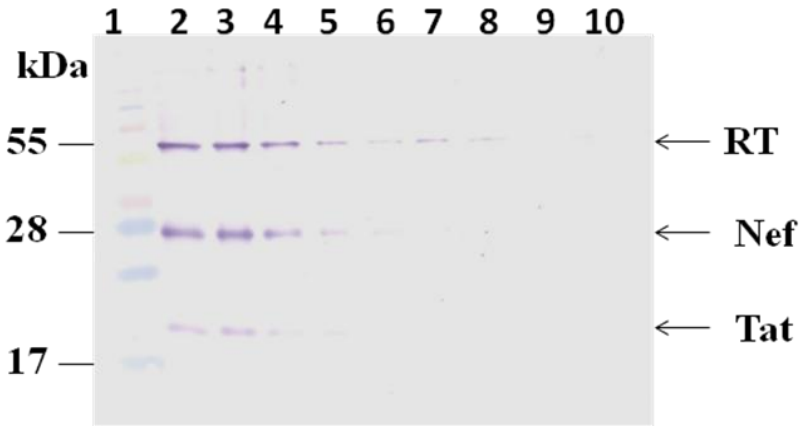

Figure 4: 2 Western blot assay for the establishment of optimum amount of proteins in a cocktail form. Purified Tat, Nef and RT proteins were prepared in cocktails containing 2-fold serial dilutions and loaded onto 12.5% electrophoresis gel. The proteins were bound to the membrane and detected with anti-Histag antibody. **Lane 1**: Multicolour broad range protein ladder, **Lane 2**: Cocktail 1: containing 0.022 µg Tat, 0.035 µg Nef & 0.06 µg RT, **Lane 3**: Cocktail 2: containing 0.011 µg Tat, 0.018 µg Nef & 0.03 µg RT, **Lane 4**: Cocktail 3: containing 0.006 µg Tat, 0.009 µg Nef & 0.015 µg RT, **Lane 5**: Cocktail 4: containing 0.003 µg Tat, 0.0045 µg Nef & 0.0075 µg RT, **Lane 6**: Cocktail 5: containing 0.002 µg Tat, 0.00023 µg Nef & 0.004 µg RT, **Lane 7**: Cocktail 6: containing 0.001 µg Tat, 0.001 µg Nef & 0.002 µg RT, **Lane 8**: Cocktail 7: containing 0.0005 µg Tat, 0.0005 µg Nef & 0.001 µg RT, **Lane 9:** Cocktail 8: containing 0.011 µg Tat, 0.00025 µg Nef & 0.0005 µg RT, **Lane 10**: Cocktail 9: containing 0.00025 µg Tat, 0.00013 µg Nef & 0.0003 µg RT.

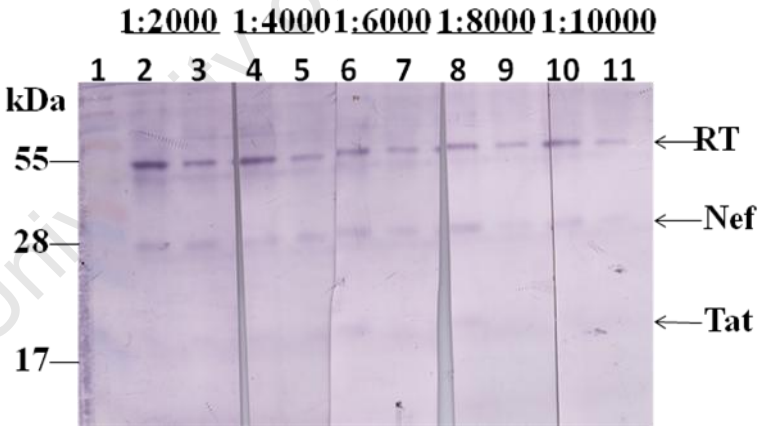

Figure 4: 3 Western blot assay for establishment of the optimum dilution of secondary antibody. Purified Tat, Nef and RT antigens were diluted to 2-fold concentrations in a cocktail and loaded onto 12.5% electrophoresis gel. The proteins were bound to the membrane that was cut into strips. The strips were incubated with positive HIV control sample and detected with 2-fold serial dilutions of anti-human secondary antibody. **Lane 1:** Multicolour broad range protein ladder, **Lanes 2, 4, 6, 8 & 10**: Cocktail 1 contained 0.022 µg Tat, 0.035 µg Nef & 0.06 µg RT, **Lanes 3, 5, 7, 9 & 11:** Cocktail 2 contained 0.011 µg Tat, 0.018 µg Nef & 0.03 µg RT**.** Numbers on the top of the Western blot represent dilution concentrations of the secondary antibody.

4.6.2 Detection of HIV-1 Tat, Nef and RT antibodies in human sera by Western blot assay

Purified recombinant Tat, Nef and RT proteins were used as antigens to detect the prevalence of antibodies against these proteins in 481 serum samples from HIV-1 infected individuals. Each serum from HIV-1 positive showed an individual response pattern, reacting with one, two or all three recombinant proteins on the strips at different levels of intensity. A representative Western blot assay is depicted in Figure 4.4.

The prevalence of antibodies to recombinant Tat, Nef and RT antigens in the sera from individuals with various CD4 counts and viral loads are shown in Table 4.1. The prevalence of antibodies to the antigens was classified into eight various categories and is summarized in Table 4.1. Antibodies to all three antigens (Tat, Nef and RT) were detected in 29 (6%) of 481 sera samples from HIV-1 infected individuals with various CD4 counts. None of the serum samples had positive responses to both Tat and Nef antigens in the absence of anti-RT responses. Similarly, none of the samples tested positive for Nef alone or Tat alone in the absence of anti-RT responses. Antibodies to Nef and RT antigens but not to Tat were detected in 191 (40%) of 481 HIV positive sera samples. A prevalence of 2% was detected for anti-Tat and anti-RT antibodies but not anti-Nef antibodies. Two hundred and ten of 481 HIV positive sera samples had anti-RT antibodies but not anti-Nef and anti-Tat antibodies. Thirty seven (8%) of 481 sera samples were negative for all three antigens. Thus, an overall of 444 of 481 sera samples from HIV-infected individuals tested positive for HIV antibodies, indicating a sensitivity of 92% for the Western blot assay (calculated as shown in section 4.2.3). At the same time, 20 of 20 HIV negative sera from healthy individuals tested negative for HIV antibodies, indicating a specificity of 100%.

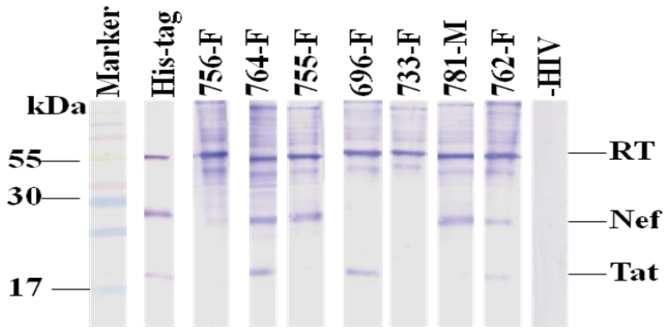

Figure 4.4: Representative Western blot assay showing the antibody responses to recombinant Tat, Nef and RT antigens in the sera of HIV-1 infected individuals. A cocktail containing equal molar concentration of Tat, Nef and RT antigens was migrated onto 12.5% SDS-PAGE and the antigens were electroblotted to the membrane. The membrane was cut into strips and used to screen the presence of HIV-1 antibodies with sera from HIV-1infected individuals. Each strip indicates the antibody responses to recombinant Tat, Nef and RT antigens. Strip labelled His-tag was bound with a cocktail containing Tat, Nef and RT antigens which were detected with anti-Histag antibody. A –HIV labelled strip was bound with a cocktail containing Tat, Nef and RT antigens which were detected with HIV negative serum and served as negative control.

Table 4.1: Frequency of anti-HIV-1 antibodies in the serum samples from HIV-1 infected individuals

		Presence of positive antibodies (%) to:							
		Tat protein: +	+	-	+	+	-	-	-
		Nef protein: +	+	+	-	-	+	-	-
		RT protein: +	-	+	+	-	-	+	-
CD4 Category (cells no/µl)	**n**								
Group A: CD4 500 or more	**115**	**7(6)**	**0(0)**	**33(29)**	**2(2)**	**0(0)**	**0(0)**	**67(58)**	**6(5)**
Group B: CD4 201 to 499	**286**	**19(7)**	**0(0)**	**128(45)**	**5(2)**	**0(0)**	**6(2)**	**101(35)**	**27(9)**
Group C: CD4 200 or less	**80**	**3(4)**	**0(0)**	**30(38)**	**1(1)**	**0(0)**	**0(0)**	**42(53)**	**4(5)**
Total:	**481**	**29(6)**	**0(0)**	**191(40)**	**8(2)**	**0(0)**	**6(1)**	**210(44)**	**37(8)**
Viral Load Category (RNA copies/ml)									
1.VL 50 to 500	**20**	**1(5)**	**0(0)**	**5(25)**	**1(5)**	**0(0)**	**1(1)**	**11(55)**	**1(5)**
2. VL 501 to 5000	**56**	**1(2)**	**8(14)**	**16(29)**	**2(4)**	**1(2)**	**0(0)**	**24(43)**	**4(7)**
3. VL 5001 to 20, 000	**77**	**6(8)**	**0(0)**	**35(45)**	**0(0)**	**0(0)**	**2(3)**	**29(38)**	**5(6)**
4. VL 20001 or more	**129**	**11(9)**	**0(0)**	**47(36)**	**0(0)**	**0(0)**	**0(0)**	**64(50)**	**7(5)**
Total:	**282**	**19(7)**	**8(3)**	**103(37)**	**3(1)**	**1(0.4)**	**3(1)**	**128(45)**	**17(6)**
Controls (HIV-samples)	**20**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**20**

4.6.2 Statistical analysis

The Western blot analysis indicated that 7% of 115 of serum samples in group A (CD4: 500 cells/µl or more), 8.4% of 286 of the serum samples in group B (CD4: 201 to 499 cells/µl) and 5% of 80 serum samples in group C (CD4: 200 cells/µl or less) had anti-Tat antibodies (Table 4.2). A two-tailed Fisher's exact test showed that the frequency of the positive responses between groups A & B, groups B & C and groups A & C were not significantly different (Table 4.2; p= 0.689, p= 0.474 and p= 0.764). The analyses showed that all the *p-value*s between various viral loads were also found to be far greater than 0.05, indicating that there were no significant differences between the groups (Table 4.2). An overall amount of 7.5% of the serum samples from HIV-1 infected individuals had anti-Tat antibodies. The frequency of antibody responses seemed to decrease in individuals having less CD4 count and higher viral load (Table 4.2).

The prevalence of antibody responses showed that 39 of 115 serum samples (33.9%) in group A, 156 of 286 (54.5%) serum samples in group B and 33 of 80 (41.3%) serum samples from HIV-1 infected individuals in group C reacted with recombinant Nef antigen (Table 4.3). The prevalence of anti-Nef antibodies in group B (CD4: 201 to 499 cells/µl) is significantly higher than the prevalence in groups A and C. However, the frequency of anti-Nef antibodies was not significantly different between groups A and C (Fishers' exact test, p= 0.366). Also the prevalence of anti-Nef antibodies between various levels of viral load was not significantly different (Table 4.3). In overall 47.4% of the serum samples from HIV-1 infected individuals had anti-Nef antibodies. Serum samples from group B (CD4: 201 to 499 cells/µl) and group 3 (VL: 501 to 20 000 RNA copies/ml) had the highest prevalence of anti-Nef antibody (Table 4.3).

Further analysis indicated that 106 of 115 (92.2%) serum samples in group A, 257 of 286 (89.9%) serum samples in group B and 76 of 80 (95%) serum samples in group C had anti-RT antibodies (Table 4.4). The prevalence of antibodies against RT antigen was not significantly different between groups A, B and C (Table 4.4). The frequency of anti-RT antibodies in groups with various levels of viral load was significantly different between only groups 3 & 4 and 2 & 4 (Table 4.4; Fishers' exact test: p= 0.0002 and p= 0.0001, respectively). This shows that the prevalence of

anti-RT antibodies is significantly higher in individuals with very high viraemia (VL: 20001 RNA copies/ml or more) than those with moderate viraemia (VL: 5001 to 20000 RNA copies/ml). Overall, 91.3% of HIV positive individuals had anti-RT antibodies.

Additional analysis looked at the prevalence of antibodies to recombinant Tat, Nef and RT antigens in the serum samples of HIV-infected heterosexual partners. As shown in Table 4.5, the frequency of anti-Tat antibodies between the sera samples from heterosexual partners was 8.9% and 9.7% for male and female individuals, respectively. The prevalence of anti-Nef antibody was 44.3% and 43.6% for female and male individuals, respectively, while those for anti-RT antibodies were 91% and 96% (Table 4.5). There were no significant differences in the frequency of antibodies between male and female heterosexual partners (Fishers' exact test: p= 0.829; p= 1.000; p= 0.195 for anti-Tat, anti-Nef and anti-RT, respectively).

Table 4.2: Anti-Tat antibody frequency in the sera of HIV-1 infected individuals with various levels of CD4 and viral load.

CD4 Categories (cells no/µl)	n	Tat positive response in (%)	Tat negative response in (%)	*p values* by Fisher's exact test
Group A: CD4 500 or more	**115**	**8 (7)**	**107 (93)**	**A vs B: p = 0.689**
Group B: CD4 201 to 499	**286**	**24 (8.4)**	**262 (91.6)**	**B vs C: p = 0.473**
Group C: CD4 200 or less	**80**	**4 (5)**	**76 (95)**	**A vs C: p = 0.765**
Total	**481**	**36 (7.5)**	**445 (92.5)**	
Viral load (RNA copies/ml)				
1. VL 50 to 500	**20**	**2(10)**	**18 (90)**	**1 vs 2 = 0.602**
2. VL 501 to 5000	**56**	**3(5.4)**	**53(94.6)**	**2 vs 3 = 0.239**
3. VL 5001 to 20 000	**77**	**9(11.6)**	**68(88.4)**	**3 vs 4 = 0.474**
4. VL 20001 or more	**129**	**11(8.5)**	**118(91.5)**	**1 vs 3 = 1.000**
				1 vs 4 =0.687
Total	**282**	**25(8.9)**	**257(91.1)**	**2 vs 4 =0.558**

Table 4.3: Anti-Nef antibody frequency in the sera of HIV-1 infected individuals with various levels of CD4 and viral load.

CD4 Categories (cells no/µl)	n	Nef positive response in (%)	Nef negative response in (%)	*p values* by Fisher's exact test
Group A: CD4 500 or more	115	39 (33.9)	76 (66.1)	A versus B =0.0002
Group B: CD4 201 to 499	286	156 (54.5)	130 (45.5)	B versus C =0.043
Group C: CD4 200 or less	80	33 (41.3)	47 (58.7)	A versus C =0.366
Total	481	228 (47.4)	253 (52.6)	
Viral load (RNA copies/ml)				
1. VL 50 to 500	20	7(35)	13(65)	1 vs 2 = 0.438
2. VL 501 to 5000	56	26(46.4)	30(53.6)	2 vs 3 = 0.867
3. VL 5001 to 20 000	77	44(57.1)	33(42.9)	3 vs 4 = 0.066
4. VL 20001 or more	129	58(45)	71(55)	1 vs 3 = 0.621
				1 vs 4 = 0.084
Total	282	135(47.9)	147(52.1)	2 vs 4 = 0.183

Table 4.4: Anti-RT antibody frequency in the sera of HIV-1 infected individuals with various levels of CD4 and viral load.

CD4 Categories (cells no/µl)	n	RT positive response in (%)	RT negative response in (%)	*p values* by Fisher's exact test
Group A: CD4 500 or more	115	106 (92.2)	9 (7.8)	A vs B =0.574
Group B: CD4 201 to 499	286	257 (89.9)	29 (10.1)	B vs C =0.189
Group C: CD4 200 or less	80	76 (95)	4(5)	A vs C =0.565
Total	481	439(91.3)	42(9)	
Viral load (RNA copies/ml)				
1. VL 50 to 500	20	18 (90)	2 (10)	1 vs 2 = 1.000
2. VL 501 to 5000	56	51 (91.1)	5 (8.9)	2 vs 3 = 1.000
3. VL 5001 to 20 000	77	69 (89.6)	8 (9.4)	3 vs 4 = 0.0001
4. VL 20001 or more	129	85(65.9)	44(34.1)	1 vs 3 = 1.000
				1 vs 4 = 0.036
Total	282	223 (79.1)	59(20.9)	2 vs 4 = 0.0002

Table 4.5: Comparison of the prevalence of antibodies to HIV-1 Tat, Nef and RT antigens in the sera of HIV-1 infected heterosexual partners.

Antibodies to:	Gender	n	Positive response	Negative response	p-values (Fishers' exact test)
Tat	F	124	11 (8.9)	113 (91.1)	F vs M = 0.829
	M	124	12 (9.7)	112 (90.3)	
Total		248	23 (9.3)	225 (90.7)	
Nef	F	124	55 (44.3)	69 (55.7)	F vs M = 1.000
	M	124	54 (43.6)	70 (56,4)	
Total		248	109 (43.9)	139(56.0)	
RT	F	124	113 (91)	11 (9)	F vs M = 0.195
	M	124	119 (96)	5 (4)	
Total		248	232 (94)	16 (7)	

4.6.1 DISCUSSION

It is well known that most antibodies against structural proteins (Gag, Pol (RT) and Env) are more readily detected in higher frequencies than those targeting regulatory (Tat and Rev) and accessory (Nef, Vif, Vpr and Vpu) proteins (Reiss *et al*., 1990; Letvin and Walker, 2003). This may explain the exclusion of regulatory and accessory proteins in most current commercial HIV detection kits and diagnostic tools. HIV-1 recombinant Tat, Nef and RT proteins play an important role in pathogenesis of HIV-1 infection by promoting virus replication (Chang *et al.,* 1994; Havlir *et al.,* 1996).

4.6.2 Optimisation of the Western blot assay

The Western blot assay was successfully optimized by establishing equal banding pattern of Tat, Nef and RT antigens and optimum titration of anti-Human IgG alkaline phosphatase conjugate. Molar equivalents of 0.011 µg Tat, 0.018 µg Nef and 0.03 µg RT (which were the minimal concentration that could still be detected) in cocktail preparation was found to be optimum for these Western blot assays, indicating that the purification process resulted in production of recombinant proteins of high purity. The transfer efficiency of Tat, Nef and RT proteins was determined by staining the SDS-PAGE gel with Coomassie blue after transfer of proteins to the PVDF membrane. It appeared that the transfer efficiency of Tat antigen was poor compared to transfer efficiency of Nef and RT proteins. This may have occurred due to the fact that the transfer efficiency can be as low as 10% with semi-dry blotting method especially when using hydrophobic proteins such as Tat (Bio-rad semi-dry booklet, USA). During optimisation, a lot of background was observed (data not shown). However, this problem was solved by incubating the sera with *Salmonella* cell lysates. This process proved to be beneficial in getting rid of the reactive antibodies to *Salmonella* proteins which is a common gut pathogen (Dykhuizen *et al*., 1996). In addition, it did not appear to affect the signal intensity of the bands for Tat, Nef and RT antigens. Indeed, overnight pre-incubation of sera with the *Salmonella* cell lysates at 4°C degrees was observed to be more effective than 2-hour incubation.

4.6.2 Detection of HIV-1 antibodies to recombinant Tat, Nef and RT antigens by Western blot assay

The performance of a Western blot assay is an important consideration when estimating the effectiveness of the test in detecting the presence of specific antibodies or antigens of interest. This may be estimated by calculating the specificity and sensitivity of the assay. In this study, sera from HIV-1 uninfected individuals were used in parallel with sera from HIV-1 infected individuals to act as negative controls as well as for estimating the specificity of the test. All the negative control sera were non-reactive in the Western blot assay, indicating specificity (proportion of actual negative which are correctly identified) of 100% for the assay. The sensitivity (proportion of actual positive which are correctly identified) of this Western blot assay was calculated as 92% in detecting the HIV-1 antibodies to Tat, Nef or RT antigens. However, this level of sensitivity may be an under-estimate because the presence or absence of antibodies to these recombinant proteins could not be confirmed using a method of known sensitivity. Also, it is possible to get HIV-1 positive sera in which anti-RT, anti-Nef and anti-Tat antibodies are absent or below the detection limit of Western blot assay. Thus, it is almost impossible for a Western blot based on only these three proteins to achieve a sensitivity of 100%. Moreover, considering reactivity to RT alone, which is the only enzymatic protein incorporated in the assay, an overall prevalence of 91% was achieved. Therefore, an overall sensitivity of 92% and a specificity of 100% were considered to be of a good performance for the optimized Western blot assay. However, this assay is still not suitable for use as a diagnostic test due to it lower sensitivity as compared ELISA HIV test (positive Rapid HIV antibody test). The sensitivity of Rapid HIV antibody test is 99.6% and this test was used to diagnose HIV infection in individuals whose sera samples were used in the current study.

4.6.2 Statistical analysis of the prevalence of antibodies to recombinant Tat, Nef and RT antigens to determine association with viral load or CD4 counts

Rezza *et al*., (2005) reported that antibodies against Tat are associated with slow progression of AIDS disease in HIV-1 infected individuals. However, antibodies to HIV-1 regulatory proteins including Tat are detected at a lower frequency in individuals with AIDS (Chou *et al*., 1988; Reiss *et al*., 1990). The reported

prevalence of anti-Tat antibodies (detected using Western blot or/and ELISA) in the sera of HIV-infected individuals ranged from 16% to 40% (Barone *et al.*, 1986; Krone *et al.*, 1988; McPhee *et al.*, 1988). This percentage is relatively higher than what is found in the current study (5% to 8.4%). The lower frequency of anti-Tat antibodies in this study were similar to those reported previously (Rezza *et al.*, 2005). The lower frequency of anti-Tat antibodies in current study may be due to shuffling of amino acids, which was done to inactivate the Tat protein. Although the shuffling of Tat protein preserved the T- cell epitopes and resulted with a robust of T-cell responses in vaccinated mice (Burgers *et al.*, 2006), it may have resulted in loss of some antigenic sites in the recombinant Tat protein. Another possible reason may be due to a change in the conformational structure of Tat protein by denaturation buffer that impeded the Western blot assay to detect the antibodies that could have been detected. Statistical analysis showed that there was no significant difference in the prevalence of anti-Tat antibodies in the sera from HIV-1 infected individuals with different CD4 and viral loads categories. The prevalence of anti-Tat antibodies was low in the serum samples of individuals with low viral load (VL: 501 to 5000 RNA copies/ml). However, there was no association between the anti-Tat antibodies with viral load or CD4 counts.

A 47.4% of sera tested had anti-Nef antibodies and these results were similar to those reported previously (Ranki *et al.*, 1987; Allan *et al.*, 1985). However, this percentage was relatively lower than some of the other studies reported previously (Wieland *et al.*, 1990; Chen *et al.*, 1999; Bahraoui *et al.*, 1990; Reiss *et al.*, 1989). These studies reported that 86% (Wieland *et al.*, 1990), 77% (Chen *et al.*, 1999), and 70% (Bahraoui *et al.*, 1990) of serum samples from HIV-1 infected individuals had anti-Nef antibodies. This difference of the prevalence of anti-Nef antibodies in the serum samples used in the current study and other related studies is possibly due to differences on the HIV-1 subtype in which the Nef protein was derived as well as the sensitivity and specificity of its method of detection. The prevalence of anti-Nef antibodies was significantly higher in group B than in groups A and C. This may have occurred due to high level of immune system activation by high HIV replication during this stage in which the immune system is not yet suppressed as a result of CD4 cell loss (Albequerque *et al.*, 2007). This increases the production of antibodies to viral proteins including Nef. This finding is supported by the low frequency of

anti-Nef antibodies in the sera samples from individuals with high CD4 count and low viral load. However, decreasing levels of the anti-Nef antibody have been associated with progression of the disease (Chen *et al*, 1999). In the current study, strong association between the high prevalence of anti-Nef antibodies with a CD4 count range of CD4: 201 to 499 cells/µl was observed.

RT protein is expressed early in HIV infection and a number of studies have reported high quantities of antibodies to recombinant RT protein (Chen *et al*., 1999; DeVico *et al*., 1988; Laurence *et al.*, 1987; Isaguliants *et al.*, 2000; Jonckheer *et al.*, 2001), explaining its potential for usage in kits for HIV-1 diagnosis. In the current study, the HIV-1 infected individuals tested had relatively high prevalence of anti-RT antibodies regardless of the CD4 count and plasma viral load levels. This showed that RT is a good diagnostic protein. The percentage was higher in relative to previous study, in which the detection of anti-RT antibodies were detected in the sera of HIV-1 subtype B infected individuals (DeVico *et al.*, 1988). This may suggest that it is possible that the detection of anti-RT antibodies also depend on the HIV-1 subtype in which the RT protein was derived. High prevalence of antibodies to RT protein is due to the fact that RT is an enzymatic protein and thus it is needed to transcribe the viral DNA to RNA during HIV infection (Mitsuya, *et al*., 1990). Other key factors that may be accountable for high prevalence of anti-RT antibodies include the fact that RT protein is more immunogenic and it has more antigenic epitopes (di Marzo-Veronese *et al*., 1986; DeVico *et al.*, 1988).

In order to determine whether there was difference in the prevalence of antibodies in the sera of HIV-1 infected heterosexual partners, the data was analysed by Fisher's exact test. There was no significance differences between the prevalence of positive responses to these antigens in the serum of heterosexual partners (anti-Tat: p=0.829; anti-Nef: p=1.000; anti-RT: p=0.195). These suggest that the presence of antibody responses to HIV-1 Tat, Nef and RT antigens were not gender dependent. Despite the unavailability of antiretroviral therapy record, some of tested serum samples were obtained from individuals that known to have received antiretroviral treatment. This may have influenced the prevalence of antibodies making it unrepresentative of natural of HIV infection.

In this study, antibodies to recombinant *Salmonella enterica* serovar Typhimurium produced HIV-1 Tat, Nef and RT antigens were detected in the sera of HIV-1 infected individuals. A prevalence of 7.5%, 47.4% and 91.3% of antibodies to Tat, Nef and RT, respectively, were detectable. There was a strong association between the high prevalence of anti-Nef antibodies with a CD4 count range of 201 to 499 cells/µl. However, there were no associations between anti-Tat or anti-RT antibodies with any clinical stage. On the basis that antibodies to RT antigen were frequently detected in the sera of HIV-infected individuals, it may be valuable to be included in diagnostic kits. However, Tat and Nef proteins may not serve a valuable role in HIV detection kits due to their low prevalence in the sera of HIV-infected individuals.

CHAPTER 5

EVALUATION OF ANTIBODY RESPONSES IN SERA OF MACAQUES VACCINATED WITH CANDIDATE DNA AND MVA HIV-1 VACCINES

CONTENTS

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CONTENTS

4.6.1 INTRODUCTION

Heterologous prime-boost immunization involves the use of two or more different vaccines expressing the same or related vaccine immunogens (Donnely *et al.,* 2005; Liu, 2003; Schneider *et al.,* 1998). One of the vaccines is used to prime the immune system while the other is used subsequently for boosting. Such a combination immunization strategy is aimed at induction and enhancement of the immune response by avoiding the anti-vector immunity which might be induced by repeated booster immunizations with the same vaccine (Donnely *et al.,* 2005; Liu, 2003; Schneider *et al.,* 1998). It improves the immune responses better than homologous prime boost which involves repeated vaccination with the same vaccine (Schneider *et al.,* 1998). The combination of plasmid DNA vaccine with recombinant protein in heterologous prime-boost immunization can induce and boost humoral responses as well as Th1 and CD8+ cellular responses (Castaldello *et al*., 2006; Darrah *et al.*, 2007; Kumer *et al*., 2006). Humoral responses have been induced with plasmid DNA vaccine boosted with the recombinant protein vaccine for HIV-1 (Barnett *et al*., 1997; Fuller *et al*., 1997). Increased level of binding and neutralizing antibodies against HIV-1 gp120 was achieved in mice and guinea pigs vaccinated with DNA vaccine encoding the *gp120* gene (Barnett *et al*., 1997). The level of antibody responses was increased by boosting with recombinant gp120 protein subunit (Barnett *et al.*, 1997). Strong gp120-specific IgG titers was also observed in rhesus macaques vaccinated with DNA vaccine encoding HIV-1 *gp120* and *p24* genes and boosted with recombinant gp120 and p24 subunit vaccines (Fuller *et al.,* 1997).

A DNA vaccine can also be combined with MVA or other viral vectors for induction and enhancement of CD4+ and CD8+ T cell responses. The DNA prime with viral vector boost induces high levels of cell-mediated responses far better than when only one vaccination is used (Wu *et al*., 2005). This occurs because it facilitates the recognition of additional epitopes of T-cell responses (Wu *et al.,* 2005). High levels of CD8+ T cells were induced in mice and monkey models vaccinated with DNA and boosted with MVA as compared to either DNA or MVA alone (Robinson *et al*., 2006). There was a reduction of severity of SIV infection in macaques vaccinated with DNA and boosted with recombinant adenovirus vaccine, indicating that the

vaccination regimen resulted in preservation of CD4+ T-cell populations (Mattapallil *et al*., 2006). A DNA vaccine expressing HIV-1 *env* and g*ag-protease* genes derived from HIV-1 subtype C in a prime-boost combination with recombinant MVA has been reported to induce high levels of immune responses in vaccinated mice (Kumar *et al*., 2006).

Burgers *et al*., (2006 & 2008) from the University of Cape Town's HIV Vaccine Research group have developed a DNA and MVA vaccines expressing HIV-1 subtype C multigene polyprotein (Gag, RT, Tat and Nef) and Env gp150. The genes were derived from HIV-1 subtype C strains Du422 and Du151 and the two vaccines were designated SAAVI DNA-C/C2 and SAAVI MVA-C respectively. SAAVI DNA-C comprised of pTHr.grttnC and pTHr.gp150CT plasmids. The pTHr.grttnC expresses HIV-1 subtype C *gag, rt, tat, nef* genes whereas pTHr.gp150CT expressed a truncated *gp150* (Burgers *et al*., 2006). SAAVI DNA-C2 comprised of pVRCgrttnC and pVCRgp150CT plasmids and expressed the same genes as SAAVI DNA-C. The only difference is that the genes were cloned in different vector (pVCR) (Burgers *et al.*, 2006). Burgers *et al*. (2006) showed that vaccination of mice with SAAVI DNA-C resulted in generation of robust IFN (interferon)-γ-producing CD8+ T cells in response to stimulation with RT peptides. In SAAVI MVA-C, the polyprotein, grttn, and the truncated gp150 are inserted into two different sites to produce a double recombinant MVA (Burgers *et al*, 2008). These vaccines have been reported to be highly immunogenic when given in DNA prime-MVA boost combination and shown to generate high frequencies of HIV-specific CD4+ and CD8+ cells in small laboratory animal models (mice and guinea pigs) and baboons (Burgers *et al*., 2008 & 2009; Shephard *et al*., 2008). Similar results were obtained in rhesus macaques vaccinated with same vaccines (unpublished data).

4.6.1 OBJECTIVE

As previously reported, binding antibodies to HIV-1 subtype C gp120 were detectable in the sera of guinea pigs and baboons primed with SAAVI DNA-C/C2 and boosted with SAAVI MVA-C vaccines (Burgers *et al*., 2009; Shephard *et al.*, 2008). The current study was aimed at evaluating humoral responses induced in rhesus macaques by the same vaccines. Thus, the objective of this study is to investigate the prevalence of HIV-1 antibodies against recombinant Tat, Nef and RT

antigens in the sera of macaques vaccinated with SAAVI DNA-C and SAAVI MVA-C by Western blot assay.

4.6.1 MATERIALS

Eleven serum samples from rhesus macaques vaccinated with SAAVI-DNA-C/C2 and SAAVI-MVA-C expressing HIV-1 subtype C polyprotein containing Gag, RT Tat, Nef (Grttn) and Env antigens were provided by Dr Wendy Burgers (Division of Medical Virology, UCT). The vaccination schedule for the macaques with the DNA and MVA vaccines is shown in Table 5.1.

Table 5.1: The vaccination schedule for macaque vaccination with SAAVI DNA-C/C2 and SAAVI MVA-C boosting

Label of Macaques sera	Pre-bleed before Vaccination (-controls)	*SAAVI DNA C/C2 vaccination Schedule	SAAVI MVA-C boost Vaccination Schedule	Time of taking sera after MVA boosting
1. P18C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 7
2. P23C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
3. P24C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
4. P27C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
5. P37C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
6. P41C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
7. P45C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
8. P67C	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 7
9. P3C2	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 7
10. P28C2	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 3
11. P30C2	✓	Weeks 0, 4 & 8	weeks 16 & 20	Week 7

*SAAVI DNA C and SAAVI DNA C2 express the same genes (*gag, rt, tat, nef* and *gp150*) but the only difference is that the genes were cloned in different vectors.

4.6.1 METHODS

4.6.2 Secondary antibody titration for detection of antibodies in macaque sera

To establish the optimum dilution of secondary antibody for detection of vaccine-induced HIV-1 antibodies in the macaque sera, a cocktail containing Tat (0.011 µg), Nef (0.018 µg), and RT (0.03 µg) proteins were subjected to 12.5% SDS-PAGE as previously described (Chapter 2, section 2.4.11). The proteins were electroblotted onto a PVDF membrane (Amersham, UK) as described previously (Chapter 2, section 2.4.13). The macaque serum was incubated with *Salmonella* cell lysates overnight at 4°C to remove antibodies to *Salmonella* cell extracts. The PVDF membrane was cut into strips and incubated with macaque sera at a dilution of 1:100 for 2 hrs on a shaker at room temperature. Anti-monkey IgG secondary antibody conjugated to alkaline phosphatase (Sigma, Germany) was diluted in various dilution (1: 2000; 1: 4000; 1: 6000; 1: 8000 and 1: 10,000). The strips were washed four times (10 min each) with washing buffer (Appendix A: 30) to remove unbound antibodies and incubated at serial dilutions of anti-monkey IgG secondary antibody conjugated to alkaline phosphatase (Sigma, Germany) for 60 min. The strips were washed 4 times at room temperature and the detection was done by developing with NBT-BCIP substrate solution for 15 min. After the reaction was stopped by rinsing with water, the strips were dried with paper towel and scanned (CanonScan 8000F, China).

4.6.2 Detection of the HIV-1 antibodies in macaque sera by Western blot assay

A cocktail of recombinant Tat (0.011 µg), Nef (0.018 µg) and RT (0.03 µg) proteins used for Western blotting experiments was subjected to 12.5% SDS-PAGE at 80 V for 2 hrs as described previously (Chapter 2, section 2.4.11). The proteins were electrophoretically transferred on to PVDF membranes as previously described (Chapter, sections 2.4.13). The membranes were blocked with 5% blocking buffer (Appendix A: 30) for 2 hrs at room temperature and left at -4^{0}C on an overnight shaker. The membranes were dried and kept at 4^{0}C until use. The night before the detection, the macaques sera (both before vaccination and after vaccination with DNA and MVA) were incubated with *Salmonella* cell lysates at dilution of 1:100 overnight at 4^{0}C to remove the antibodies to *Salmonella enterica* serovar Typhimurium. The membranes were taken out of the fridge, cut into thin strips, labelled and soaked in methanol (for 2 min) and water for 5 min. After soaking, the strips were washed once for 10 min with washing buffer (Appendix A: 31) and each strip was incubated with serum at a dilution of 1:100 at room temperature for 2 hrs.

The sera from macaques before DNA vaccination were used as negative controls. The binding of macaque antibodies was detected by goat anti-monkey IgG conjugated to alkaline phosphatase (Sigma, Germany) at a dilution of 1:8000 for 60 min. The strips were washed 4 times with washing buffer (Appendix A: 30) and visualized with NBT-BCIP substrate reaction following the manufacturer's instructions (Roche Diagnostics, Germany). The strips were left in NBT-BCIP substrate reaction for 15 min, rinsed with water and dried with paper towel. The strips were taped on a white, A4 paper and scanned (CanonScan 8000F, China).

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4.6.1 RESULTS

4.6.2 Establishment of the best anti-Monkey IgG titration for detection of antibodies in macaque sera

The concentration of secondary antibody to detect the macaque sera was established by comparing the intensity of the reactive bands with serial dilutions at 1:2000. 1:4000, 1: 6000, 1:8000 and 1:10 000. The results confirmed that concentration of secondary antibody at 1:8000 and 1:10 000 dilution showed best detection with no background (Figure 4.1, strip 9 & 11). However, only antibodies to recombinant RT protein were detected but not to recombinant Nef and Tat proteins (Figure 5.1, strips 2, 4, 6, 8, & 10). Expectedly, there were no antibody reaction observed in strips incubated with sera from macaques before vaccination and served as negative controls (Figure 5.1, strips 3, 5, 7, 9 & 11). This indicated that there were no antibody responses to Nef and Tat protein in tested macaque sample.

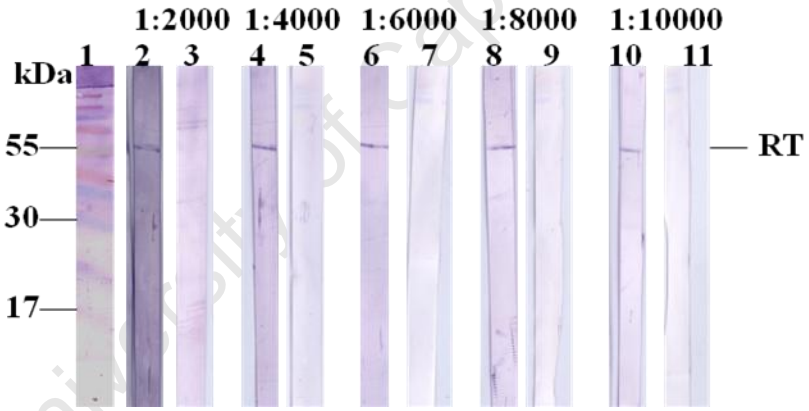


Figure 5.1: Titration of secondary antibody (anti-monkey IgG) for detection of the macaque sera. A cocktail containing Tat, Nef and RT antigens were separated on 12.5% electrophoresis gel, transferred to the membrane that was cut into strips. The antigens were reacted with sera from macaques vaccinated with DNA and MVA vaccines and sera from macaques before vaccination was used as a negative control. The Tat, Nef and RT antigens were visualized with various titrations of anti-monkey IgG secondary antibody linked with alkaline phosphatase and NBT/BCIP substrate. **Strips 1**: Multicolour broad range protein ladder. **Strips 2, 4, 6, 8 & 10:** Strips bound with cocktail contained Tat, Nef and RT antigens which were detected with sera of macaques vaccinated with DNA and boosted with MVA expressing HIV-1 subtype C Grttn & env. (No bands were observed for Nef and Tat antigens because there were no detectable antibody responses to these antigens for this particular sample). **Strips 3, 5, 7, 9 & 11:** Strips bound with cocktail contained Tat, Nef and RT antigens which were detected with pre-vaccination sera of macaques to act as negative control sera.

4.6.2 Detection of HIV-1 Tat, Nef and RT antibodies in macaque sera

The prevalence of HIV-1 antibodies against recombinant Tat, Nef and RT antigens were investigated in the sera of vaccinated macaques with SAAVI-DNA-C/C2 vaccine and SAAVI-MVA-C expressing HIV-1 subtype C Gag, RT, Tat, Nef and Env proteins. Out of 11 serum samples from macaques vaccinated with DNA vaccine and boosted with MVA, only 4 (36%) had anti-Tat antibodies (Figure 5.2, strips 2, 6, 8 and 14). Three of 11 (27%) macaque sera had antibodies against recombinant Nef antigen (Figure 5.2, strips 2, 6, and 14). However, anti-Nef antibody responses in two of these sera were very weak and resulted in the development of very faint bands on the blot for Nef antigen (Figure 5.2, strips 2, and 6). Despite of low intensity, 5 (40%) sera of macaques had anti-RT antibodies. Three of the positive macaque sera were obtained at week 3 post the second SAAVI MVA-C vaccination (Figure 5.2, strips 2, 6 & 8) and 1 was obtained at week 7 post the second SAAVI MVA-C vaccination (Figure 5.2, strips 14). Three bands on Figure 4.2 (strip 24) indicate recombinant Tat, Nef and RT antigens which was detected with penta anti-His antibody and served as positive control. No antibody responses were detectable in the pre-immunization sera of the macaques, suggesting a high specificity for the assay. A few serum samples showed some background that may have resulted from non-specific reactivity.

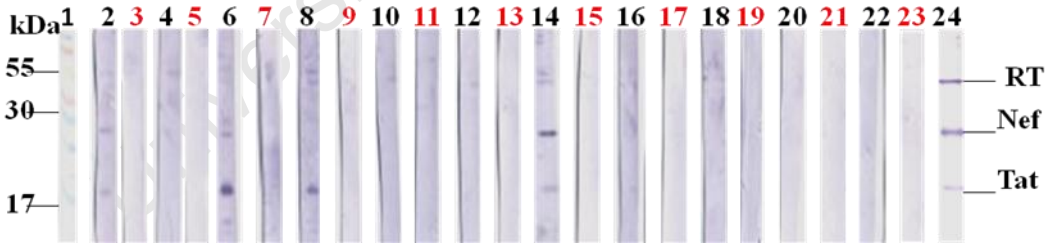


Figure 5.2: Western blot assay showing the prevalence of HIV-1 (Tat, Nef and RT) antibodies in sera of macaques vaccinated with SAAVI-DNA and SAAVI-MVA. A cocktail containing Tat, Nef and RT antigens were separated on 12.5% electrophoresis gel, transferred to the membrane that was cut into strips. The antigens were reacted with sera of macaques vaccinated with DNA vaccine and boosted with MVA vaccines and sera of macaques before vaccination and used as a negative control. The Tat, Nef and RT antigens were visualized with anti-monkey IgG secondary antibody linked with alkaline phosphatase and NBT/BCIP substrate. Each strip indicates antibody response to HIV-1 Tat, Nef and RT antigen. **Black coloured strips 2, 4, 6, 8 10, 12, 14, 16, 18, 20, 22:** Strips bound with cocktail contained Tat, Nef and RT antigens and detected with sera of macaques vaccinated with DNA and boosted with MVA expressing HIV-1 subtype C Grttn & env. **Red coloured strips 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 & 23:** Strips bound with cocktail contained Tat, Nef and RT antigens and detected with sera of macaques before vaccination with DNA

and MVA. **Strips 24:** Positive control strip bound with cocktail contained Tat, Nef and RT antigens and detected with penta anti-His antibody.

4.6.1 DISCUSSION

Development of HIV-1 vaccine that could induce both humoral and cell mediated responses still remains a great challenge. Combination of DNA and MVA based vaccines are important for inducing the T cell (CD8+ and CD4+) and humoral responses against various pathogens such as HIV (Liu, 2005). Previous studies showed that a DNA prime with an MVA boost induces higher magnitudes of both humoral and cell mediated responses than either vaccine alone (Kent *et al*., 1998; Schneider *et al.*, 1998). Abundant and high quality CD4+ and CD8+ T- cell responses were induced by a prime-boost combination of DNA and MVA vaccines in mice, non-human primates and human (Burgers *et al*., 2008 & 2009; Cosma *et al*., 2003; Hirsch *et al*., 1996; Shephard *et al*., 2008; Sutter *et al*., 1994). In the current study, the presence of antibodies against recombinant Tat, Nef and RT antigens were investigated in the sera of macaques vaccinated with DNA and MVA vaccines expressing Gag, Tat, RT, Nef and Env antigens in a prime-boost immunization strategy.

4.6.2 Detection of antibodies to Tat, Nef and RT antigens in sera of macaque vaccinated with SAAVI DNA-C/C2 and boosted with SAAVI MVA-C

The results obtained by Western immunoblot assay revealed only 36%, 27% and 40% of the vaccinated macaques generated detectable antibodies against HIV-1 recombinant Tat, Nef and RT antigens, respectively. Some of the bands for the reactivity of one or more antigens (Tat, Nef and RT) were very faint, suggesting presence of low level of antibodies in the tested sera. This was despite the finding that high frequencies of T-cell responses to all the five vaccine imunogens, including Tat, Nef, and RT, were generated in the peripheral blood of the vaccinated animals (Burgers *et al*, unpublished). The prevalence of antibody responses to Tat, Nef and RT antigens in the sera of macaques is lower relative to the prevalence of antibody responses in the sera of HIV-1 infected individuals. A possible explanation is that the immune system of HIV-1 infected people is continuously exposed to viral antigens, thus resulting to high level of detectable anti- Tat, -Nef and -RT antibodies. In contrast, macaques were exposed to HIV-1 antigens expressed by the DNA and MVA vectors for only five times through vaccinations.

There are other factors which may have affected weak or absence of binding antibody responses to HIV-1 subtype C Tat, Nef and RT antigens in the sera of macaques vaccinated with SAAVI DNA-C/C2 and SAAVI MVA-C vaccines. One possible explanation is that although MVA-vector vaccines are excellent in boosting T-cell responses to heterologous antigens, they are very poor in inducing or boosting humoral responses to the same antigens (Mwau *et al*., 2004). This explained the low or absence of binding antibodies in the sera of vaccinated macaques in the current study. This, combined with the fact that DNA vaccines themselves are weak inducers of humoral responses, may have resulted in undetectable levels of antibodies being generated, leading to low prevalence of Tat, Nef and RT antibodies. DNA vaccine boosted with protein based vaccine is an effective way of inducing strong binding antibody responses against the HIV-1 viral proteins (Richmond *et al,* 1998). They produce a better quality of humoral responses because they produce properly folded and glycosylated proteins (Wang *et al.*, 1993). They can also recognise more antigenic epitopes as compared to viral vector vaccines (Wang *et al*., 2005). Previous studies demonstrated a high magnitude of antibody responses in mice, rabbits and rhesus macaques vaccinated with DNA boosted with protein subunit vaccines (Barnett *et al*., 1997; Doria-Rose *et al*., 2005; Fuller *et al*., 1997).

In conclusion, this study showed a low prevalence of antibody responses to HIV-1 subtype C Tat, Nef and RT antigens in the sera of macaques vaccinated with SAAVI DNA-C/C2 and SAAVI MVA-C vaccines. The sera was tested at a dilution of 1/100 and it is possible that more positive sera may have been detected at lower serum dilution. In addition, this study demonstrated that a Western blot assay based on purified HIV-1 Tat, Nef and RT proteins is a useful research tool in detection of binding antibodies to corresponding vaccine immunogens in macaque sera. Thus, future work to measure the performance of this Western blot test and validation for use in HIV vaccine research would be justifiable.

CHAPTER 6

GENERAL CONCLUSIONS

The aim of the study was to develop a recombinant *Salmonella enterica* serovar Typhimurium expressing HIV-1 subtype C Tat, Nef and RT proteins. The recombinant HIV-1 subtype C Tat, Nef and RT were purified and used in Western blot assay to detect antibodies to the proteins in the sera of HIV-1 subtype C infected individuals from Cape Town, South Africa and macaques vaccinated with candidate HIV-1 vaccines, SAAVI-DNA-C/C2 and SAAVI-MVA-C. The following five general conclusions were drawn from the study:

6.1 Recombinant HIV-1 Tat, Nef and RT proteins were produced from *Salmonella enterica* serovar Typhimurium.

Recombinant *Salmonella* containing pGEM+Tat, pGEM+Nef and pGEM+RT plasmids expressed high levels of HIV-1 Tat, Nef and RT respectively. Codon optimization of the genes, fusion of the genes to the *LacZa* and use of the *lac* promoter were among the factors that facilitated the high production of the antigens by the bacteria.

6.2 Recombinant HIV-1 Tat, Nef and RT proteins were purifed from *Salmonella enterica* serovar Typhimurium.

HIV-1 Tat, Nef and RT were purified from the recombinant *Salmonella enterica* serovar Typhiumurium. Only Nef, but not Tat and RT could be purified under native conditions. This showed that only Nef was expressed as a soluble protein and Tat and RT were expressed as inclusion bodies. All the three antigens were purified in bulk under denaturing conditions. This suggested that even Nef was expressed mainly as inclusion bodies. The His-tag facilitated the purification of the HIV-1 Tat, Nef and RT proteins by affinity chromatography. It was concluded that *Salmonella* was a good heterologous bacterial expression system which could be used to produce foreign proteins in large quantities.

6.3 Antibodies to HIV-1 Tat, Nef and RT were detected in sera of HIV-1 infected individuals

HIV-1 subtype C Tat, Nef and RT antigens purified from recombinant *Salmonella enterica* serovar Typhimurium were capable of detecting HIV-1 anti-Tat, anti-Nef and anti-RT antibodies in the sera of HIV-1-infected individuals by Western blot method. Sera from 91% of the HIV-positive individuals reacted with RT, while only 47.4% and 7.5% of the HIV-postive individuals reacted to Nef and Tat respectively. Low prevalence of anti-Tat antibodies may be due to shuffling of Tat amino acids that was done to inactivate the Tat protein. Antibodies to all the three proteins was detected in 6% of the sera while 42% and 45% of the sera had antibodies to two and one (principally to RT) proteins respectively. The levels of antibodies to Tat, Nef and RT in the sera of the individuals probably varied as the intensities of the Western blot bands were observed to vary. There was a strong association between the high prevalence of anti-Nef antibodies with one of the clinical stage (CD4 count of 201 to 499 cells/µl). However, there were no associations of anti-Tat and anti-RT antibodies with CD4 counts or viral load. The observation that antibodies to RT antigen were frequently detected in the sera of HIV-infected individuals suggests a possible application of purified RT proteins to improve current HIV diagnostic kits.

6.4 Antibodies to HIV-1 Tat, Nef and RT were detected in sera of macaques vaccinated with HIV vaccines.

HIV-1 Tat, Nef and RT antigens purified from the recombinant *Salmonella enterica* serovar Typhimurium were further shown to be reactive to HIV-1 anti-Tat, anti-Nef and anti-RT antibodies respectively in sera of macaques previously vaccinated with HIV-1 subtype C vaccines expressing Gag, RT, Tat, Nef and Env antigens. The purified Tat, Nef and RT reacted with only 36%, 27% and 40% respectively of sera of vaccinated animals. This suggested that the macaques did not produce enough antibodies against DNA vaccine probably because MVA was poor in boosting the antibody responses. However, this study demonstrated that a Western blot assay based on purified HIV-1 Tat, Nef and RT proteins is a possible useful research tool in detection of binding antibodies to corresponding vaccine immunogens in macaque sera.

6.5 Further studies need to be done in future

Since the *Salmonella enterica* serovar Typhimurium developed in this study produced high levels of the HIV antigens, further studies are needed to show whether the bacteria can be used as a HIV vaccine vector. It further needs to be investigated to check whether the purified proteins can be used as HIV subunit vaccines. The purified proteins also need to be investigated on their suitability for use as immunological reagents for assays in HIV vaccine research.

APPENDIX A
MEDIA, SOLUTIONS AND REAGENTS
BACTERIAL AND SOLUTIONS

1. 2x Yeast Tryptone (2YT) Agar

Tryptone	16 g
Yeast extracts	10 g
NaCl	5 g
Agar	15 g
DH_2O	1000 ml

The mixture was autoclaved and upon cooling, the supplements or antibiotics were added and poured into plates to solidify.

2. 2x YT media

Tryptone	16 g
Yeast extracts	10 g
NaCl	5 g
DH_2O	900 ml

The pH was adjusted to 7.0 with NaOH, made up the volume to 1000ml with dH_2O and autoclaved.

3. 100 mg/ml Ampicillin

Ampicilin	1 g
DH_2O	10 ml

The mixture was filter sterilized and stored at -70°C in 100ul aliquots.

4. 100 mg/ml IPTG

IPTG	1 g
DH_2O	10 ml

The mixture was filter sterilized and stored at -70°C in 100ul aliquots.

5. 100x Aromix compounds

4- aminobenzoic acid	200 mg
2.3-Dihydroxyl benzoic acid	200 mg
L-phenylalanine	800 mg
L-tryptophan	800 mg
DH_2O	200 ml

The mixture was filter sterilized and stored at -70°C in 30 ml aliquots.

6. 100x Tyrosine

L-Tyrosine	800 mg
0.1M HCL	200 ml

The mixture was filter sterilized and stored at -70°C in 30 ml aliquots.

BUFFERS FOR PREPARATION OF COMPETENT CELLS

7. 10% Glycerol stock

Glycerol	10 ml
DH_2O up to	100 ml

Autoclaved and stored at 4°C.

8. 1 M Calcium chloride

$CaCl.\ 2H_2O$	29.4 g
DH_2O up to	200 ml

Autoclaved and stored at 4°C.

SOLUTIONS AND GEL PREPARATION FOR ELECTROPHORESIS GEL

9. 0.5 M EDTA, pH 8.0

EDTA	93.1 g
DH_2O to	100 ml

The pH was adjusted to 8.0 and volume made up to 300 ml and stored at room temperature.

10. 1X Tris-Borate-EDTA (TBE) Buffer

Tris-HCl	269 g
Boric acid	137.6 g
EDTA, pH8.0	18.63g
DH_2O	25L.

This is usually prepared for the entire lab users in 25 L bottle and stored at room temperature.

11. 1.5% agarose gel

Agarose powder	7.5 g
DH_2O	500 ml

The mixture was melted on a microwave and ethidium bromide was added to a total concentration of 2 µl/ml and stored at 37°C.

SOLUTIONS FOR ISOLATION OF PLASMID DNA

12. 10X Solution I stock (Resuspension solution):

1 M Tris-HCl pH 8.0	25 ml
20% Glucose	45.5 ml
0.5 M EDTA pH 8.0	20 ml
DH_20 up to	100 ml

The solution was stored at room temperature.

13. Solution II (Lysis solution)

10 N NaOH	2 ml
25% SDS (w/v)	10 ml
DH_20	100 ml

The solution was usually prepared prior use.

14 Solution III (Neutralization solution)

K-acetate	147 g
DH_20	250 ml

The pH of the solution was adjusted with acetic acid to 4.8 and the volume was made up with dH_20 up to 500 ml, autoclaved and stored at 4°C.

15 70% Ethanol

Ethanol	70 ml
DdH_20	100 ml

Stored at room temperature.

16 Tris-EDTA (TE) Buffer pH 9.0

1 M Tris HCl	2 ml
1 M EDTA	200 ul
DH_20	150 ml

The pH of the solution was adjusted to 9.0 and the volume made up to 200 ml. Stored at room temperature.

BUFFERS AND GEL PREPARATION FOR SDS-PAGE GEL

17 Acrylamide/bis (30% T, 2.67%C)

Acrylamide	87.6 g
N'N'-bis –methylene-acrylamide	2.4 g
DH_20	300 ml

Since acrylamide is a neurotoxin, it was weighed out with gloves on and a gas mask. The solution was filter sterilized and stored at 4°C.

18 1.5M Tris-HCL, pH 8.8

Tris base	27.33 g
DH_20	100 ml

The pH was adjusted 8.8 with 6 N HCL and volume made up to 150 ml with distilled water and stored at 4°C.

19 0.5 M Tris-HCL, pH 6.8

Tris base	9 g
DH_20	100 ml

The pH was adjusted 6.8 with 6N HCL and volume made up to 150ml with distilled water and stored at 4°C.

20 10% SDS

SDS	10 g
DH_20	80 ml

The mixture was mixed and the volume was made up to 10 0ml and stored at room temperature

21 2X Laemmli loading dye (SDS reducing agent)

Distilled water	3.8 ml
0.5 M Tris-HCL, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% bromophenol blue	0.4 ml
	8.0 ml

Stored at 4°C.

22 12.5% Resolving gel preparation-0.375 M Tris, pH 8.8

Distilled water	3.35 ml
1.5 M Tris-HCL, pH 8.8	2.5 ml
10% SDS stock	100 ul
Acrylamide 30%	4.0 ml
10% Ammonium persulphate (Freshly made)	50 ul
TEMED	5.0 ul
	10 ml

Add all reagents except APS and TEMED and degas under vaccum for 15 min. To initiate polymerization add APS and TEMED and swirl gently to mix.

23 4% Stacking gel preparation- 0.126 M Tris, pH 6.8

Distilled water	6.1 ml
0.5 M Tris-HCL, pH 6.8	2.5 ml
10% SDS stock	100 µl
Acrylamide/bis (30% stock)	1.33 ml
10% Ammonium persulphate (Freshly made)	50 ul
TEMED	10 ul
	10 ml

Add all reagents except APS and TEMED and degas under vaccum for 15 min. To initiate polymerization add APS and TEMED and swirl gently to mix.

24 10X SDS-PAGE Running Buffer

Tris-HCl	30.3 g
Glycine	144 g
SDS	10 g
DH_2O	1000 ml

The buffer was stored at 4°C

25 1X SDS-PAGE Running Buffer

10X SDS-PAGE Running buffer	100 ml
DH_2O	900 ml

The buffer was stored at 4°C

BUFFERS FOR PROTEIN EXTRACTION, PURIFICATION AND WESTERN BLOT

26 Protein lysis buffer

10% SDS	60 ml
50% Glycerol	40 ml
1 M Tris-base, pH 6.8	12.5 ml
DH_2O to	200 ml

The buffer was stored at room temperature.

27 1 M Tris-HCL, pH 8.0

Tris-base	36.3 g
DH_2O	150 ml

The pH of the buffer was adjusted to 8.0 and volume made up to 200 ml and stored at room temperature.

28 2 M Sodium Chloride (Nacl)

Nacl	58.44 g
DH_2O to	500 ml

The buffer was stored at room temperature.

29 1 M Tris, ph 7.5

Tris-base	36.3 g
DH_2O	150 ml

The pH of the buffer was adjusted to 7.5 and volume made up to 200 ml and stored at room temperature.

30 Blocking/Diluent/Washing buffer

Fat Free Milk	40 g
1 M Tris pH 7.2	40 ml
2 M Nacl	150 ml
Tween 20	5 ml
DH_2O	700 ml

The mixture was allowed to dissolve and made up to 1L with distilled water.

31 Substrate

NBT/BCIP	1pellet
DH_2O	10 ml

The mixture was allowed to dissolve prior use.

32 10X Transfer Buffer

Tris-HCl	30.3 g
Glycine	144 g
DH_2O	1000 ml

The buffer was stored at 4°C

33 1X Transfer Buffer

10X Transfer buffer	100 ml
Methanol	200 ml
DH_2O	600 ml

The pH of the buffer was checked prior use and if necessary the pH was adjusted to 8.3.

34 Coomassie blue staining solution

Coomassie brilliant blue	1 tablet
Methanol	400 ml
Glacial acetic acid	70 ml
DH_2O	530 ml

The solution was wrapped with foil and stored at 4°C.

35 Destaining solution

Methanol	200 ml
Glacial acetic acid	70 ml
DH_2O	730 ml

The solution was prepared under vacuum and stored at room temperature.

36 1 M NaH_2PO_4

NaH_2PO_4	5.58 g
DH_2O to	200 ml

The solution was stored at 4°C.

37 1 M Tris-base

Tris-base	0.25 g
DH_2O to	400 ml

The solution was stored at 4°C.

38 Denaturing Lysis buffer (buffer B)

NaH_2PO_4 (1 M)	20 ml
Tris-base (1 M)	2 ml
Urea (8 M)	96.9 g
DH_2O	100 ml

The urea was dissolved and the pH was adjusted to 8.0 and the volume made up to 200 ml with distilled water. This was prepared freshly all the time.

39 Denaturing Wash buffer (buffer C)

NaH_2PO_4 (1 M)	20 ml
Tris-base (1 M)	2 ml
Urea (8 M)	96.9 g
DH_2O	100 ml

The urea was dissolved and the pH was adjusted to 6.3 and the volume made up to 200 ml with distilled water. This was prepared freshly all the time.

40 Denaturing Elution buffer (buffer E)

NaH_2PO_4 (1M)	20 ml
Tris-base (1M)	2 ml
Urea (8M)	96.9 g
DH_2O	100 ml

The urea was dissolved and the pH was adjusted to 4.5 for elution of 10-30 kDa proteins and 3.7 for 50-100 kDa proteins. The volume made up to 200 ml with distilled water and this was prepared freshly all the time.

41 Native Lysis buffer

NaH_2PO_4. H_2O (50 mM)	6.9 g
NaCl (300 mM)	17.54 g
Imidazole (10 mM)	0.68 g
DH_2O	100 ml

The mixture was dissolved and the pH was adjusted to 8.0 with NaOH and the volume made up to 200 ml with distilled water and stored at 4 ^{0}C.

42 Native Wash buffer

NaH_2PO_4. H_2O (50 mM)	6.9 g
NaCl (300 mM)	17.54 g
Imidazole (20 mM)	1.36 g
DH_2O	100 ml

The mixture was dissolved and the pH was adjusted to 8.0 with NaOH and the volume made up to 200 ml with distilled water and stored at 4 ^{0}C.

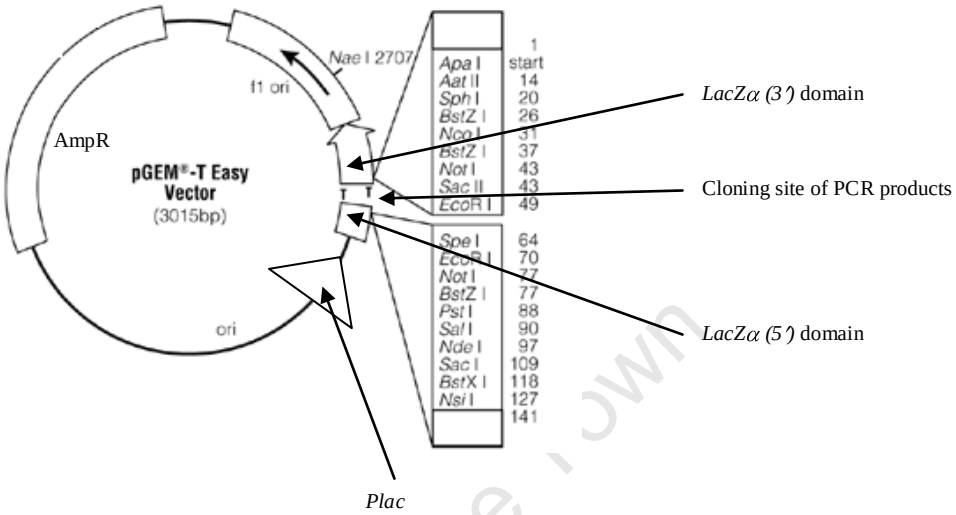
43 Native Elution buffer

NaH_2PO_4. H_2O (50 mM)	6.9 g
NaCl (300 mM)	17.54 g
Imidazole (250 mM)	17 g
DH_2O	100 ml

The mixture was dissolved and the pH was adjusted to 8.0 with NaOH and the volume made up to 200 ml with distilled water and stored at 4 ^{0}C.

APPENDIX B
PLASMID MAP

1. pGEM-TEASY VECTOR MAP



The pGEM(R)-T Easy Vector has been linearized with EcoRV at base 60 of this sequence and a T added to both 3' –ends (adapted from Promega, USA catalogue). This vector is designed for cloning PCR products. The pGEM-T Easy vector has a mutated version *pMB1* origin of replication and this makes the plasmid to have a copy number of 300-400 per bacterial cell. The pMBI origin of replication is closely related to that of *CoIE1*. The *CoIE1* origin of replication makes a plasmid to have a copy number of 300-500 per cell.

DNA & PROTEIN SEQUENCES

2. HisTat

```
1       ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1        M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61      CATCATCATCATCATCACATCGAAGGTCGTGGCGCCCATATGCTGGGCATTAGCTATGGC
21       H  H  H  H  H  H  I  E  G  R  G  A  H  M  L  G  I  S  Y  G

121     CGCAAAAAACGTCGTCAGCGTCGTAGCACCCCGCCGAGCAGCGAAGATCATCAGAACCCG
41       R  K  K  R  R  Q  R  R  S  T  P  P  S  S  E  D  H  Q  N  P

181     ATTAGCAAACAGCCGCTGCCGCAGACCCGTGGCGATCCGACCGGCAGCGAAGAAAGCAAA
61       I  S  K  Q  P  L  P  Q  T  R  G  D  P  T  G  S  E  E  S  K

241     AAAAAAGTCGAAAGCAAAACCAAAACCGATCCGTTTGATTGCAAATATTGCAGCTATCAC
81       K  K  V  E  S  K  T  K  T  D  P  F  D  C  K  Y  C  S  Y  H

301     TGCCTGGTGTGCTTTCAGACCAAAGGTCTGGGTATCTCCTACGGTCGTAAAAAGCGCATG
101      C  L  V  C  F  Q  T  K  G  L  G  I  S  Y  G  R  K  K  R  M

361     GAACCGATTGATCCGAATCTGGAACCGTGGAATCATCCGGGCAGCCAGCCGAATACCCCG
121      E  P  I  D  P  N  L  E  P  W  N  H  P  G  S  Q  P  N  T  P

421     TGCAACAAATGCTATTGTAAGTACTGTTCCTACCATTGCTTGGTCGACTAA
141      C  N  K  C  Y  C  K  Y  C  S  Y  H  C  L  V  D  *
```

3. HisNef

```
1          ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1           M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61         CATCATCATCATCATCACATCGAAGGTCGTGGCGCCCATATGGTGGGCTGGCCGGCGGTG
21          H  H  H  H  H  H  I  E  G  R  G  A  H  M  V  G  W  P  A  V

121        CGTGAACGTATTCGTCGTACCGAACCGGCGGCGGAAGGCGTGGGCGCGGCGAGCCAGGAT
41          R  E  R  I  R  R  T  E  P  A  A  E  G  V  G  A  A  S  Q  D

181        CTGGATAAACATGGCGCGCTGACCAGCAGCAATACCGCGCATAACAATCCGGATTGCGCG
61          L  D  K  H  G  A  L  T  S  S  N  T  A  H  N  N  P  D  C  A

241        TGGCTGCAAGCGCAGGAAGAAGAACCGGAAGTCGGCTTTCCGGTCCGTCCGCAGGTGCCG
81          W  L  Q  A  Q  E  E  E  P  E  V  G  F  P  V  R  P  Q  V  P

301        CTGCGTCCGATGACCTATAAAGCGGCGTTTGATCTGAGCTTTTTTCTGAAAGAAAAAGGC
101         L  R  P  M  T  Y  K  A  A  F  D  L  S  F  F  L  K  E  K  G

361        GGCCTGGAGGGCCTGATCTATAGCAAAAAACGCCAGGATATTCTGGATTTATGGGTCTAT
121         G  L  E  G  L  I  Y  S  K  K  R  Q  D  I  L  D  L  W  V  Y

421        CATACCCAGGGCTATTTTCCGGATTGGCAGAATTATACCCCGGGTCCGGGCGTGCGTCTG
141         H  T  Q  G  Y  F  P  D  W  Q  N  Y  T  P  G  P  G  V  R  L

481        CCGCTGACCTTTGGCTGGTGCTTTAAACTGGTGCCGGTCGATCCGGAAGAAGTGGAAGAA
161         P  L  T  F  G  W  C  F  K  L  V  P  V  D  P  E  E  V  E  E

541        GCGAACAAAGGCGAAAACAACTGTCTGCTGCATCCGCTGAGCCAGCATGGCATGGAAGAT
181         A  N  K  G  E  N  N  C  L  L  H  P  L  S  Q  H  G  M  E  D

601        GCGGATCGCGAAGTCCTGAAATGGGTCTTTGATAGCAGCCTGGCGCGTCGTCATCTGGCG
201         A  D  R  E  V  L  K  W  V  F  D  S  S  L  A  R  R  H  L  A

661        CGTGAAAAACATCCGGAATATTATAAAGATGCATAA
221         R  E  K  H  P  E  Y  Y  K  D  A  *
```

4. HisRT

```
1          ATGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC
1           M  T  M  I  T  P  S  Y  L  G  D  T  I  E  Y  S  S  Y  A  S

61         CATCATCATCATCATCACATCGAAGGTCGTGGCGCCCATATGGGGCCCATTAGCCCGATT
21          H  H  H  H  H  H  I  E  G  R  G  A  H  M  G  P  I  S  P  I

121        GAAACCGTGCCGGTGAAACTGAAACCGGGCATGGATGGCCCGAAAGTCAAACAGTGGCCG
41          E  T  V  P  V  K  L  K  P  G  M  D  G  P  K  V  K  Q  W  P

181        CTGACCGAAGTGAAAATTAAAGCGCTGACCGCGATTTGCGAAGAAATGGAAAAAGAAGGC
61          L  T  E  V  K  I  K  A  L  T  A  I  C  E  E  M  E  K  E  G

241        AAAATCACCAAAATCGGCCCGGAAAACCCGTATAACACCCCGATCTTTGCGATCAAAAAA
81          K  I  T  K  I  G  P  E  N  P  Y  N  T  P  I  F  A  I  K  K

301        GAAGATAGCACCAAATGGCGTAAACTGGTGGATTTTCGCGAACTGAACAAACGCACCCAG
101         E  D  S  T  K  W  R  K  L  V  D  F  R  E  L  N  K  R  T  Q

361        GATTTTTGGGAAGTCCAACTGGGCATTCCGCATCCGGCGGGCCTGAAGAAAAAGAAAAGC
121         D  F  W  E  V  Q  L  G  I  P  H  P  A  G  L  K  K  K  K  S

421        GTGACCGTGCTGGATGTCGGCGATGCGTATTTTAGCGTGCCGCTGGATGAAGGCTTTCGC
141         V  T  V  L  D  V  G  D  A  Y  F  S  V  P  L  D  E  G  F  R

481        AAATATACCGCGTTTACCATCCCGAGCATCAACAATGAAACCCCGGGCATTCGCTATCAG
161         K  Y  T  A  F  T  I  P  S  I  N  N  E  T  P  G  I  R  Y  Q
```

```
541       TATAACGTGCTGCCGCAGGGCTGGAAAGGCAGCCCGGCGATTTTTCAGGCGAGCATGACC
181        Y  N  V  L  P  Q  G  W  K  G  S  P  A  I  F  Q  A  S  M  T

601       AAAATTCTGGAACCGTTTCGCGCGAAAAACCCGGAAATCGTGATCTATCAGTATATGGCG
201        K  I  L  E  P  F  R  A  K  N  P  E  I  V  I  Y  Q  Y  M  A

661       GCGCTGTATGTGGGCAGCGATCTGGAAATTGGCCAGCATCGCGCGAAAATTGAAGAACTG
221        A  L  Y  V  G  S  D  L  E  I  G  Q  H  R  A  K  I  E  E  L

721       CGCGAACATCTGCTGAAATGGGGCTTTACCACCCCGGATAAAAAACACCAGAAAGAACCG
241        R  E  H  L  L  K  W  G  F  T  T  P  D  K  K  H  Q  K  E  P

781       CCGTTTTTATGGATGGGCTATGAACTGCATCCGGATAAATGGACCGTCCAGCCGATTCAA
261        P  F  L  W  M  G  Y  E  L  H  P  D  K  W  T  V  Q  P  I  Q

841       CTGCCGGAAAAAGATAGCTGGACCGTCAACGATATTCAGAAACTGGTGGGCAAACTGAAT
281        L  P  E  K  D  S  W  T  V  N  D  I  Q  K  L  V  G  K  L  N

901       TGGACCAGCCAGATTTATCCGGGCATTAAAGTGCGTCAGTTATGCAAACTGCTGCGTGGC
301        W  T  S  Q  I  Y  P  G  I  K  V  R  Q  L  C  K  L  L  R  G

961       ACCAAAGCGCTGACGGATATTGTCCCGCTGACGGAAGAAGCGGAACTGGAACTGGCGGAA
321        T  K  A  L  T  D  I  V  P  L  T  E  E  A  E  L  E  L  A  E

1021      AACCGCGAAATTCTGAAAGAACCTGTGCACGGCGTCTATTATGATCCGAGCAAAGATCTG
341        N  R  E  I  L  K  E  P  V  H  G  V  Y  Y  D  P  S  K  D  L

1081      ATTGCGGAAATCCAGAAACAGGGCGATGACCAGTGGACCTATCAGATCTATCAGGAACCG
361        I  A  E  I  Q  K  Q  G  D  D  Q  W  T  Y  Q  I  Y  Q  E  P

1141      TTTAAAAACCTGAAAACCGGCAAATATGCGAAACGTCGCACCACCCATACCAACGATGTG
381        F  K  N  L  K  T  G  K  Y  A  K  R  R  T  T  H  T  N  D  V

1201      AAACAACTGACCGAAGCGGTGCAGAAAATCAGCCTGGAAAGCATTGTGACCTGGGGCAAA
401        K  Q  L  T  E  A  V  Q  K  I  S  L  E  S  I  V  T  W  G  K

1261      ACCCCGAAATTTCGCCTGCCGATCCAGAAAGAAACCTGGGAAATCTGGTGGACCGATTAT
421        T  P  K  F  R  L  P  I  Q  K  E  T  W  E  I  W  W  T  D  Y

1321      TGGCAGGCGACCTGGATTCCGGAATGGGAATTTGTTAATACCCCGCCGCTGGTGAAACTG
441        W  Q  A  T  W  I  P  E  W  E  F  V  N  T  P  P  L  V  K  L

1381      TGGTATCAACTGGAAAAAGAACCGATTGCGGGCGCGGAAACGTTCCATGCATAA
461        W  Y  Q  L  E  K  E  P  I  A  G  A  E  T  F  H  A  *
```

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